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SCIENTIFIC EDITION

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

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1957 Iodine Research Award Nominations Requested Prior to January 1, 1957

Nominations are now being received by the AMERICAN PHARMACEUTICAL ASSOCIATION for the 1957 Chilean Iodine Educational Bureau, Inc., Award recognizing outstanding research in the chemistry and pharmacy of iodine and its compounds as applied in pharmacy or medicine.

The award consists of $1,000 and a diploma setting forth the reasons for selection of the recipient. It may be presented each year at the annual meeting of the ASSOCIATION.

Any member of the ASSOCIATION may propose a nominee by submitting eight copies of each of the publications to be considered in the competition, a biographical sketch of the nominee including date of birth, and a list of his publications. Eight copies of the nomination must be submitted to Robert P. Fischelis, Secretary of the AMERICAN PHARMACEUTICAL ASSOCIATION, 2215 Constitution Ave., N. W., Washington 7, D. C. To be eligible for the 1957 Award, *nominations must be received on or before January 1, 1957.*

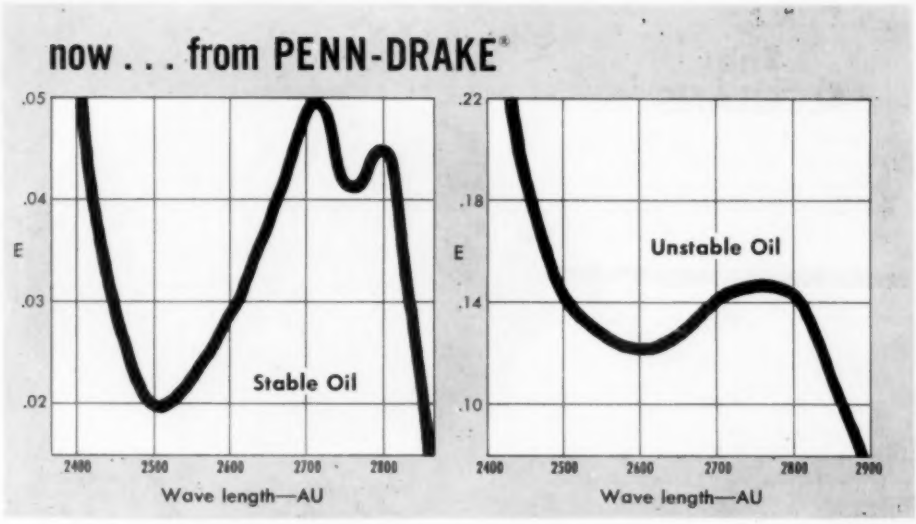
A nominee must be a resident of the United States or Canada. He must have accomplished outstanding research in the chemistry or pharmacy of iodine and its compounds as applied in pharmacy or medicine.

During the period covered by the nomination the nominee shall have been actively engaged in, shall have completed, or shall have published a report upon the line of investigation for which the award is made. During the period of two years prior to the date of nomination, the nominee shall not have been engaged in research under the sponsorship of the Chilean Iodine Educational Bureau, Inc.

The recipient will be selected by an award committee which is appointed by the chairman of the ASSOCIATION'S Council. The present committee includes Justin L. Powers, chairman; F. F. Blicke, Paul Block, E. A. Brecht, Stanley G. Mittelstaedt, Lloyd M. Parks, and Linwood F. Tice.

The recipient will deliver a paper or lecture upon the subject of his scientific work at the meeting at which the award is conferred. His paper, or address, will then be published in the JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION. Travel expense to the meeting at which the award is to be made is provided for up to $250.

The award is now in its ninth year. The recipient of the first award was Dr. William T. Salter, Yale University pharmacologist; the second recipient was Dr. George M. Curtis, Chairman of the Department of Surgical Research and Professor of Surgery at Ohio State University, and the third was Dr. C. P. Leblond, Professor of Anatomy, McGill University, Montreal, Canada. The fourth award was shared jointly by Dr. George Moore of the University of Minnesota Medical School and Dr. Moses Ashkenazy, Chief of Neurosurgery, Veterans Hospital, Houston, Tex. Dr. Vernon H. Wallingford, Mallinckrodt Chemical Works, St. Louis, Missouri, was the recipient of the fifth award. Dr. Jack Gross, Associate Professor of Anatomy, State University of New York, College of Medicine in Brooklyn, received the sixth award and Dr. Dominick Papa, Schering Corporation, Bloomfield, N. J., received the seventh award. The eighth award was conferred on Dr. John E. Christian, Professor of Pharmaceutical Chemistry, School of Pharmacy, Purdue University, Lafayette, Indiana, during the 1956 Convention of the AMERICAN PHARMACEUTICAL ASSOCIATION.



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The United States Pharmacopœial Convention, Inc.

SIXTH ANNUAL FINANCIAL STATEMENT

Based upon the Report of the Auditor for the year ended December 31, 1955

STATEMENT OF INCOME AND EXPENSE

Income:—			
Sales of pharmacopeias:			
Collections		$223,341.70	
Add—Increase in accounts receivable—			
Balance, December 31, 1955	$42,320.35		
Balance, December 31, 1954	3,634.30	38,686.05	$262,027.75
Sales of Reference Standards:			
Collections		$ 20,333.55	
Add—Increase in accounts receivable—			
Balance, December 31, 1955	$ 2,586.04		
Balance, December 31, 1954	1,740.64	845.40	
		$ 21,178.95	
Add—Reference standards collections not remitted to general account at year end		377.87	21,556.82
Interest on investments		$ 6,862.50	
Less—Amortization of bond premiums		22.65	6,839.85
Interest on savings deposits			2,494.60
Use of text by others			4,080.00
Grant-in-aid of the American Cancer Society			7,000.00
Miscellaneous			32.39
Total Income			$304,031.41
Less—Expense:—			
Revision:			
Cash disbursements		$ 67,568.16	
Less—Increase in inventory—			
Balance, December 31, 1955	$17,016.83		
Balance, December 31, 1954	15,696.66	1,320.17	$ 66,247.99
Administration			5,757.74
Publications			
Cash disbursements		$ 96,543.71	
Less—Increase in inventory			
Balance, December 31, 1955	$23,494.92		
Balance, December 31, 1954	332.00	23,162.92	73,380.79
Headquarters			
Cash disbursements		$ 4,462.94	
Add—Depreciation expense—			
Building	$ 2,430.77		
Furnishings and equipment	3,158.55	5,589.32	10,052.26
Total Expense			$155,438.78
Excess of income over expense			$148,592.63

STATEMENT OF CASH RECEIPTS AND DISBURSEMENTS

Receipts:		
Sales of pharmacopeias	$223,341.70	
Sales of reference standards	20,333.55	
Interest on investments	6,862.50	
Interest on savings deposits	2,608.19	
Use of text by others	4,080.00	
Grant-in-aid of the American Cancer Society	7,000.00	
Miscellaneous	32.39	
Total receipts	$264,258.33	

Expense:

	Revision	Administration	Publications	Headquarters	Furn. & Equip.	Total
Printing and binding			$96,543.71			$ 96,543.71
Salaries	$41,588.97	$4,482.00		$1,234.61		47,305.58
Meetings	2,913.84	575.84				3,489.68
Supplies	899.93	56.90				956.83
Postage & telegrams	1,687.62					1,687.62
Utilities				1,986.03		1,986.03
Repairs & maintenance				489.32		489.32
Insurance				752.98		752.98
General	-20,477.80	643.00			475.15	21,595.95
Total Expense	$67,568.16	$5,757.74	$96,543.71	$4,462.94	$475.15	$174,807.70
Assistant, Dr. Beal's Subcommittee		$ 233.77				
Insurance		197.77				
Library		309.36				
Reference Standards		11,115.23				
Retirement & Social Security		8,416.12				
Miscellaneous		205.55				
		$20,477.80				

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NOTICE

PAPERS FOR SECTION MEETINGS OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

The next annual meeting of the American Pharmaceutical Association will be held in New York, N. Y., April 28 through May 3, 1957. Titles of papers must be submitted to the appropriate Section secretary not later than January 15, 1957 and abstracts (not to exceed 200 words) not later than February 15, 1957, or preferably earlier, in order to assure a place on any Section program.

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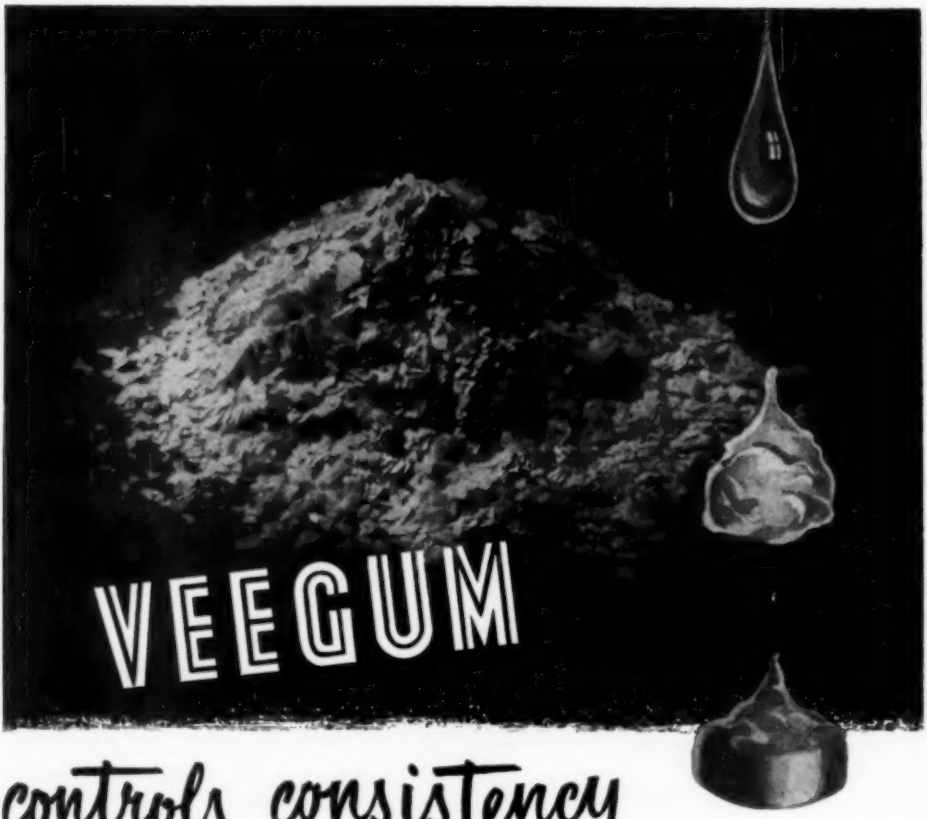

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Scientific Edition

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

VOLUME XLV — *DECEMBER 1956* — NUMBER 12 — CONSECUTIVE NO. 24

Pharmaceutical Aspects of Reserpine*

By A. F. LEYDEN, E. POMERANTZ, and E. F. BOUCHARD

Results are presented of long-term formulation and stability studies of reserpine. Procedures are described for the preparation of capsules, compressed tablets, injections, and an elixir of reserpine. Compressed tablets may be prepared either by double compression or by wet granulation. Acacia, gelatin, dextrose, sucrose, lactose, starch, dicalcium phosphate, and Carbowax 4000 and 6000 are compatible with reserpine. Magnesium stearate, calcium stearate, and talc have also been used in tablet and capsule formulations with no adverse effect on reserpine. Citric acid, tartaric acid, acetic acid, alcohol, benzyl alcohol, Tween 80, polyethylene glycol 300, and propylene glycol generally are satisfactory solvents or cosolubilizers in elixirs and injections. Reserpine can be incorporated into tablet coatings when direct combination with other active ingredients causes incompatibility, assay difficulty, or when the reserpine is needed immediately and the other ingredients are better released later. Reserpine is compatible with phenobarbital, veratrum viride, amphetamine sulfate, methyl testosterone, hyoscyamine sulfate, atropine sulfate, and niacinamide. Reserpine solutions in clear glass bottles exposed to diffuse light lost potency. Sensitivity to ultraviolet light is best prevented by storage in amber glassware.

RESERPINE is an alkaloid common to a number of Rauwolfia species, including *R. serpentina*, *R. vomitoria*, *R. heterophylla*, and *R. micrantha*. As a purified crystalline compound derived from one or more of the above root sources, reserpine has found wide medical usage since 1953.

Although a number of alkaloids isolated from Rauwolfia root have exhibited significant pharmacological effects, reserpine is the only one available in commercial quantities which duplicates, for practical purposes, the action of the whole root: (*a*) a mild, gradual and sustained lowering of blood pressure; (*b*) a tranquilizing or sedative effect (usually without hypnosis); and (*c*) a slowing of the heart rate.

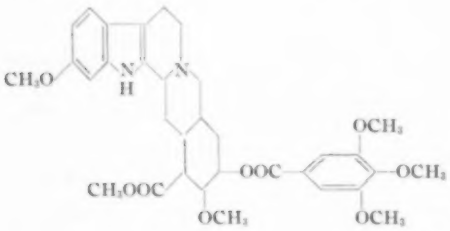
The value of reserpine as a mild hypotensive agent, effective in mild and moderate labile hypertension, has already been established. More recently the tranquilizing effect of the drug has been extended to the management of psychiatric disorders. Many reports pertaining to the usefulness of reserpine in this field have appeared in the last year.

Crystalline reserpine occurs as white to light-tan needles, insoluble in water, but moderately soluble in various organic solvents. The melting point of reserpine varies according to the method of measurement used. Values of 262–265° C. (1, 2) and 271–271.5° C. (3) have been reported.

* Received April 13, 1956, from the Pharmaceutical Section, Technical Service Department, Chas. Pfizer & Co., Inc., Brooklyn 6, N. Y.
Presented to the Scientific Section, A. PH. A., Detroit meeting, April 1956.

Reserpine possesses characteristic infrared and ultraviolet absorption spectra.

The alkaloid is a heterocyclic organic compound known to possess a system of 6 rings as shown:



$C_{33}H_{40}O_9N_2$

Reserpine is an ester alkaloid (4). Saponification with alkali yields 3, 4, 5-trimethoxybenzoic acid, reserpic acid, and methanol.

Reserpine is susceptible to decomposition by light and oxidation, properties it shares with other indole-type compounds. The material discolors quite rapidly when exposed to direct light, but this discoloration usually does not reflect a large loss in potency. Reserpine is packaged in polyethylene-lined fiber drums or in amber glass bottles as a safeguard against exposure to light. However, practical experience in handling reserpine indicates that the alkaloid is stable both in the crystalline state and as a triturate with U. S. P. lactose.

In the treatment of hypertension and in neuropsychiatric conditions reserpine usually is administered orally in the form of compressed tablets or capsules, or as an elixir; parenteral administration is by the intramuscular or intravenous route.

The extremely small quantities of reserpine incorporated in dry dosage forms designed for treatment of hypertension made a triturated form of the alkaloid desirable to facilitate handling. The first such trituration with U. S. P. lactose contained 1.0 per cent of reserpine. As success in treating psychotic conditions with higher potency reserpine tablets and capsules was reported, a triturate containing 10 per cent reserpine activity was also offered.

PROCEDURE

Triturations.—Reserpine was blended with four times its weight of U. S. P. lactose and passed through a 0.010-inch stainless steel herringbone screen using a Bantam Mikro Pulverizer or comparable equipment. The remainder of the lactose was passed through a 0.020-inch stainless steel herringbone screen. The 20% reserpine trituration was then blended with the lactose that had been passed through the 0.020-inch screen. The resulting trituration was a fine white powder.

Hard Gelatin Capsules.—To obtain a capsule containing 0.25 mg. reserpine the following formula was used:

Reserpine trituration, 1%	25 mg.
Corn Starch, U. S. P.	100 mg.
Magnesium Stearate, U. S. P.	6.3 mg.
Each capsule weighs	131.3 mg.

The reserpine trituration was incorporated with the corn starch and magnesium stearate and the resulting mixture filled into capsules.

Compressed Tablets.—Long-term stability studies show that compressed reserpine tablets can be manufactured by either wet granulation or double compression. Initial manufacturing instructions for reserpine tablets issued by our laboratories suggested using the double compression method because of the possibility of hydrolyzing the ester linkage. Early reports also indicated that reserpine when combined with certain excipients or additives tended to oxidize in the presence of circulating warm moist air.

The following formulation containing 0.25 mg. reserpine per tablet proved satisfactory regardless of the manufacturing procedure employed:

Reserpine trituration, 1%	25.0 mg.
Lactose, U. S. P.	123.8 mg.
Sucrose, U. S. P.	123.8 mg.
Corn Starch, U. S. P.	27.5 mg.
	300.1 mg.
Magnesium Stearate, U. S. P.	3.0 mg.
Each tablet weighs	303.1 mg.

Double Compression.—Reserpine trituration, the excipients, and one-half the magnesium stearate were blended until homogeneous. This mixture was compressed into large (1/2 in. to 5/8 in.) slugs and the slugs were broken up into granules by forced screening through a 10-mesh screen. The remaining magnesium stearate was added and after a short blending period the granules were compressed into tablets using 13/32 in. standard round concave punches and dies.

Wet Granulation.—The excipients were blended with the reserpine trituration and the mixture was granulated with 10% starch paste. The wet mass was forced through an 8-mesh screen, dried at 110° F. in a forced-air oven, and reoscillated through a 10-mesh screen. Magnesium stearate was added and after a short blending period, the granules were compressed into tablets using 13/32 in. standard round concave punches and dies.

The method of manufacture for higher potency reserpine compressed tablets is similar to that outlined for 0.25-mg. products. The formula for tablets containing 6.0 mg. reserpine (weighing approximately 300 mg.) uses the 10% trituration and proportions of other ingredients are altered accordingly.

Compressed reserpine tablets made by both methods had good appearance. Similarity in hardness and in disintegration rates was noted. The wet granulated tablets disintegrated in seventeen minutes in distilled water at 37° C. (Stoll-Gershberg apparatus). Double compressed tablets disintegrated in ten minutes under the same conditions. The hardness of compressed tablets made by both

methods was 9 to 11 Kg. as measured by the Monsanto Hardness Tester.

Elixir.—Reserpine is not soluble in water, but is soluble to some extent in alcohol, glycols, acetone, benzene, ethyl acetate, and carbon tetrachloride. The alkaloid is freely soluble in glacial acetic acid and in chloroform.

The photosensitivity of reserpine causes it to discolor rapidly when dissolved in benzene and other solvents. Solutions of reserpine in chloroform are particularly unstable to light and are, in addition, affected by trace impurities derived from both chloroform and reserpine.

Very few solvents can be used in the formulation of pharmaceutical reserpine solutions. As a result the use of solubilizers was investigated, and citric and tartaric acids were found very useful. Five and ten per cent aqueous solutions of these acids permit the solubilization of approximately 3 and 6 mg. per ml. of reserpine, respectively. Both acids have been used successfully in the formulation of reserpine solutions. Acetic acid, as a solvent for reserpine, also has been used in liquid reserpine formulations. A combination of solubilizers and solvents was considered the best approach to the formulation of a reserpine elixir containing 0.1 mg./ml.

Reserpine	10 mg.
Tartaric Acid, U. S. P.	500 mg.
Ethyl Alcohol, U. S. P.	10 ml.
Propylene Glycol, U. S. P.	5 ml.
Methylparaben, U. S. P.	180 mg.
Propylparaben, U. S. P.	20 mg.
Water	15 ml.
Simple Syrup, U. S. P.	70 ml.
FD&C Yellow No. 10	8 mg.
FD&C Red No. 1	1 mg.
FD&C Blue No. 4	1 mg.
Flavor	As desired
	100 ml.

Reserpine, an equal amount of tartaric acid and the mixed parabens were dissolved in ethyl alcohol (with slight heat). When the reserpine was in solution, propylene glycol was added. The balance of the tartaric acid and the mixture of dyes were dissolved separately in a minimum amount of simple syrup. These solutions when combined with the ethyl alcohol-propylene glycol solution of reserpine were mixed at 35–37° C., cooled, and flavored. A polish filtration was used before bottling.

The alcohol content of the elixir formula can be varied. Preparations containing up to 14% alcohol by volume have been marketed. Glycerin may be substituted for all or part of the propylene glycol.

The flavoring of a reserpine elixir is not difficult. Dodge and Olcott's lime mint flavor No. 8684 imparts a desirable taste and is compatible with reserpine. Since choice of flavor can vary so widely with manufacturers our laboratories have not thoroughly screened flavors combined with reserpine.

Injection.—The formulation of an injectable reserpine solution involved the same principles used in making the elixir. Benzyl alcohol, because of its bacteriostatic properties, was substituted for ethyl alcohol. To obtain a solution containing 2.5 mg. per ml. of reserpine the following formula was used:

Reserpine	250 mg.
Benzyl Alcohol, U. S. P.	2 ml.
Citric Acid, Anhydrous, U. S. P.	250 mg.
Tween 80[1]	10 ml.
Water for Injection, U. S. P.	*q. s.* to 100 ml.

Reserpine and citric acid were dissolved in benzyl alcohol (with slight heat). The Tween 80 and the water for injection were added to the benzyl alcohol, and the solution was filtered. The solution was sterilized by bacterial filtration, using a 0.03 Selas candle and then filled into 2-ml., Type I, sterile vials. The vials were sealed with neoprene stoppers and aluminum caps.

Although reserpine is not significantly soluble in Tween 80, this surface-active agent functions to stabilize the parenteral solution. When the Tween 80 is removed from the formula some of the reserpine precipitates after a short interval. Either polyethylene glycol 300 or propylene glycol may be substituted for Tween 80 in the injectable formulation. These compounds function as cosolubilizers. The *p*H of the injection was 3.0. Discoloration results when the formulation is autoclaved. Although the potency loss is small after autoclaving it is advisable to sterilize by bacterial filtration.

Reserpine in Tablet Coating.—When reserpine was first offered commercially it was apparent that it would find wide usage in specialty products, combined with other active medicinals. Several drugs considered for combination with reserpine, such as α-tocopherol and mephenesin, were expected to cause difficulty in assay. A few, such as aminophylline and theobromine, were considered likely to be incompatible with reserpine. Inclusion of reserpine in the sugar coating rather than the core appeared a logical solution to a tablet formulation involving incompatibilities or assay difficulties. This procedure was suggested until more data on the pharmaceutical aspects of reserpine were accumulated.

In developing a combination product of aminophylline (100 mg.) and reserpine (0.1 mg.) there was opportunity to investigate the stability of reserpine in the sugar coating of compressed tablets. This was also a method of achieving rapid release of the alkaloid, since it was combined with a material which traditionally has been enteric coated to render it nonirritating. This product combined the diuretic and vasodilating effects of aminophylline with the hypotensive and tranquilizing actions of reserpine.

Addition of Reserpine.—Dust-free aminophylline cores weighing three and one-half grains were enteric coated by the usual method with shellac. Ten thousand enteric-coated tablets were warmed to 40° C. A single coat of gelatin solution was applied (no dusting powder was used), and then two coats of concentrated simple syrup (25 pounds sucrose per gallon of water) heated to 40° C. were applied and dried with warm air.

The required amount of reserpine was triturated with a dusting powder consisting of 80% potato

[1] Registered trademark, Atlas Powder Company, Wilmington, Delaware.

starch and 20% talc. Sufficient powder was used to add the complete reserpine requirement in three separate applications. A 30% overage of reserpine was added to cover physical loss and to maintain at least a 10% overage in the finished tablet. The alkaloid was added to the tablets by first applying concentrated syrup (55° C.) and then immediately adding approximately one-third of the reserpine-containing dusting powder. The syrup-reserpine powder coatings were dried by directing a warm-air draft on the outside of the pan. Radiant heat was also used successfully. This syrup-powder procedure was repeated twice for a total of three applications. The reserpine was then sealed in by the application of two coats of shellac. Warm air was not directed into the pan until the shellac seal had been applied and thoroughly dried. The tablets were subcoated, smoothed, and finished.

Recent reports showed that reserpine and aminophylline are compatible. The anticipated difficulty in assaying for reserpine in products containing mephenesin and α-tocopherol materialized and was resolved by using chromatographic separation techniques before determining the reserpine content by ultraviolet absorption.

Use of a direct warm-air draft in drying the reserpine and powder coats in an early experiment caused a pronounced drop in reserpine potency, presumably as a result of oxidation. In this test a mixture of reserpine, calcium carbonate, talc, and kaolin was exposed to damp warm air. Air alone apparently was not responsible as a solution of reserpine remained stable after air had been bubbled through it. Tests showed that in a dehumidified coating room the three coats of reserpine dried quite readily without application of heat and that the overall coating time was not lengthened appreciably.

Preliminary studies revealed a tendency for heavy metals, notably iron, to form a complex with reserpine. This complex is stable and makes assay difficult. In a multivitamin-and-mineral tablet used for obese or geriatric patients, where the tranquilizing effect of reserpine would be desirable, the addition of the alkaloid in the coating avoids the complexing problem.

STABILITY

The pharmaceutical products made by previously described procedures were studied for reserpine stability under varying conditions of time and temperature (Tables I and II).

The purity of reserpine used in triturations and compressed tablets proved to be an important factor in achieving good stability. When reserpine, both purified and nonrecrystallized, was made into a 1% trituration with lactose, the purified reserpine was more stable at both temperatures studied (Table I). The purified reserpine was 98% pure and the nonrecrystallized material assayed 95.5%. The recrystallization procedure appears to remove trace impurities, decomposition products, heavy metals, and transition elements deleterious to reserpine. The color stability of triturates made from nonrecrystallized reserpine also was poor.

Complete retention of reserpine potency in hard gelatin capsules made with corn starch and magnesium stearate was observed after storage for one year at room temperature in amber glass bottles (Table I). Other excipients such as rice and tapioca starches, mannitol and talc have been used in hard gelatin capsules without adverse effects on reserpine stability.

Compressed tablets prepared by double compression and wet granulation showed excellent stability when stored at 37° C. and at room temperature in

TABLE I.—STABILITY OF RESERPINE FORMULATIONS[a]

Reserpine Preparation	12 Wk at 37° C	6 Mo. at Rm. Temp.	12 Mo. at Rm. Temp.
Trituration, 1%			
Reserpine, purified	100		100
Reserpine, noncrystallized	93		89
Capsules, hard gelatin, 0.25 mg.			100
Tablets, 0.25 mg.			
Double compressed			
Reserpine, purified	89	98	99
Reserpine, noncrystallized	78	85	74
Wet granulation			
Reserpine, purified	89	102	99
Reserpine, noncrystallized	71	78	..
Tablet coating,[b] 0.1 mg.			
On aminophylline	110[c]	90[d]	124
On placebo	110[c]	107[d]	97
Elixir, 0.1 mg./ml.	85	99	93
Injection, 2.5 mg./ml.	88		90

[a] Figures are expressed as per cent potency retained. No overage used.
[b] Figures are expressed as per cent potency retained calculated on basis of initial assay.
[c] Initial assay.
[d] Six months at 37° C.

TABLE II.—GENERAL COMPATIBILITY[a]
1 MG./GM. RESERPINE

	Drug	Concn. Gm.	3 Mo. at 50° C.	6 Mo. at Rm. Temp.
1.	Phenobarbital	32 mg.	100	106
2.	*Veratrum viride*	6 mg.	94	98
3.	*d*-Amphetamine sulfate	10 mg.	96	122
4.	Methyltestosterone	20 mg.	92	90
5.	Thiamine hydrochloride	2 mg.		
	Riboflavin	4 mg.		
	Vitamin B-12	20 mcg.	62	84
	Calcium Pantothenate	1.5 mg.		
	Vitamin A	4,000 units		
	Vitamin D	400 units		
6.	Hyoscyamine sulfate	0.2 mg.	98	96
	Atropine sulfate	0.2 mg.		
7.	Niacin	100 mg.	96	98

[a] Figures are expressed as per cent potency retained; no overage was used.

screw-cap, amber glass bottles (Table I). Poor stability of the reserpine trituration prepared with nonrecrystallized material again showed in tablets prepared by both methods.

The wet granulated tablets were prepared using starch paste. Further evaluation of the wet granulation procedure indicates that 10% acacia solution, 50% dextrose solution, 10% gelatin solution, or simple syrup also may be used. Tablets made using these solutions have been stored at room temperature for six months with no loss in reserpine potency.

Compressed tablets made by both methods (Table I) were prepared with lactose, sucrose, corn starch, and magnesium stearate. Additional study has shown mannitol, dicalcium phosphate, corn and potato starches, Carbowaxes 4000 and 6000 to be compatible with reserpine. Calcium stearate, stearic acid, and talc have been used in a number of reserpine compressed tablet formulations and are compatible with the drug.

Compressed tablets coated with reserpine and stored in screw-cap, amber glass bottles at 37° C. and at room temperature had satisfactory stability (Table I).

The distribution of reserpine in the coating of the reserpine-aminophylline preparation was poor. The relatively small number of tablets used probably accounts for the lack of homogeneity. To help overcome the variation in reserpine content, a large number of tablets were taken at each assay interval. The figures reported are averages of a large number of individual determinations.

A better distribution of reserpine was obtained when 50,000, 5-grain placebo cores were coated in the same manner. The substitution of dicalcium phosphate, talc, and gelatin solution for lactose, talc, and simple syrup in the process produced an improved coating with equivalent reserpine stability (Table I).

The elixir (Table I) was stored in screw-cap, amber bottles at 50° C. and room temperature. In view of the relatively small potency loss of reserpine in the elixir stored at both temperatures, the formulation was considered practical. The injectable solution was stored in the dark at 50° C. and at room temperature with relatively small losses in potency.

Storage of aqueous solutions of active medicinals at 50° C. is generally considered an extremely exhaustive test. The reserpine stability after three months' storage at this temperature coupled with the potency retention figures reported for compressed tablets and hard gelatin capsules stored at 37° C. show it to be a fairly heat-stable alkaloid.

Compatibility.—Anticipating the numerous combination specialty products containing reserpine that probably would be introduced, a screening program was initiated. One or two compounds from each class of materials whose combination with reserpine seemed reasonable were combined with the alkaloid to study compatibility. Wet granulated blends were stored at 50° C. and room temperature in screw-cap, amber bottles.

All blends contained 1 mg. of reserpine per Gm. of blend. The other ingredients were added in amounts usually found in individual dosage forms. The following materials were chosen: phenobarbital, veratrum viride, *d*-amphetamine sulfate, methyl testosterone, vitamins A and D, B-complex vitamins, and hyoscyamine and atropine sulfates.

Considering variation in distribution, assay error and difficulty in extracting reserpine from several of the blends, none of the materials seriously affected reserpine potency (Table II). Reserpine also exhibits satisfactory stability when combined with mephenesin, acetylcarbromal, α-tocopherol, aminophylline, and sodium salicylate.

Light Sensitivity.—In the dry state, as crystalline material or as a trituration with lactose, discoloration of reserpine occurs rapidly when exposed to direct light, but loss in potency usually is small. In solvents such as benzene or chloroform, reserpine is very sensitive to light, losing potency almost as soon as exposed. In order to determine the effect of light an aqueous solution of reserpine was prepared and stored under the following conditions: (*a*) filled in clear glass vials and stored in diffused light, (*b*) filled in amber glass vials and stored in diffused light, and (*c*) filled in amber glass vials and stored in darkness.

After six months' storage at room temperature, no appreciable color change occurred in any sample. The reserpine solution stored in clear glass vials in diffused light lost approximately 10% of its potency at this time. When stored in amber glass under the same conditions, approximately 7% potency loss was recorded. No loss occurred in the sample stored in amber glass in darkness. It may be concluded that reserpine will lose potency when exposed to light, and that visually observed color change is not a true index of the extent of decomposition.

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Physical Chemical Properties and Ultraviolet Spectral Characteristics of Amyl Nitrite*

By DALE H. SZULCZEWSKI,† MARTIN YUNKER, and T. HIGUCHI

The ultraviolet spectra of amyl nitrite, which has been determined in various polar and nonpolar solvents, exhibits rather unusual details in the long ultraviolet range. Attempts have been made to ascertain whether this behavior can be directly attributed to pure amyl nitrite or whether it is due to a persistent impurity. The apparent molar absorbance of the drug is unexpectedly low. The vapor pressure of amyl nitrite has been determined as a function of temperature by the Ramsey-Young method.

DESPITE THE FACT that amyl nitrite has been used medicinally for a good many years, relatively little information is available regarding some of its physical chemical properties. Since data of this nature were desired for a projected deterioration study, determinations were made on a purified lot of the drug. The results of these measurements are presented in this paper.

ULTRAVIOLET ABSORPTION

Thompson and Purkis (1) studying the ultraviolet absorption of nitrites in general in the vapor phase, concluded that the characteristic absorption spectra were due to the NO group when attached to an atom capable of donating electrons. No attempt was made, however, to determine the absorbances of the esters.

The ultraviolet spectra of amyl nitrite in isooctane and alcoholic solution, presented in Fig. 1, appear to be qualitatively similar to that obtained by Thompson and Purkis. The molar absorbances for the major peaks for the solvents indicated are given in Table I.

TABLE I.—MOLAR ABSORBANCES OF ISOAMYL NITRITE IN ISOOCTANE AND 95% ETHYL ALCOHOL

Solvent	Molar absorbance 344 mμ	368 mμ	356.3 mμ
Isooctane	58	53	65
95% Ethyl alcohol	53	50	62

These are rather low values for molar absorbance of organic compounds.

There are two possible explanations for such low values of molar absorbance: (*a*) the absorption curves obtained are actually due to an unknown substance which is in equilibrium with the ester or is a degradation product of the ester; (*b*) the absorption curves represent weak but real absorption characteristics of amyl nitrite. This situation would arise if the ester molecules have very poor absorption efficiency.

The first possibility may arise, for example, if the equilibrium suggested by Levy (2) for nitrite ester in the vapor phase occurred in these solutions: $R-ONO \rightleftharpoons RO+NO$.

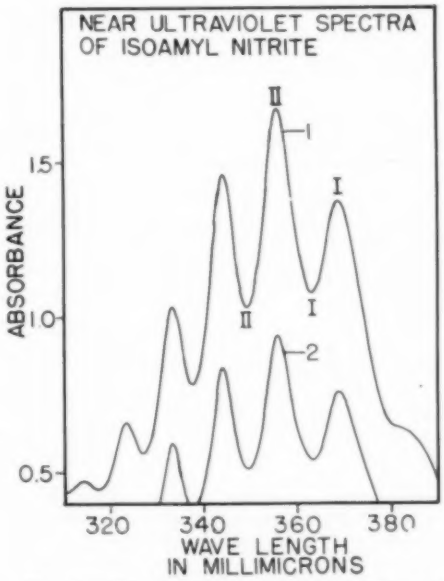

Fig. 1.—Near ultraviolet spectra of isoamyl nitrite; 1-1:400 w/v dilution of amyl nitrite in 95% ethyl alcohol; 2-1:800 w/v dilution of amyl nitrite in isooctane.

If the absorbing species is the free radical, Beer's law would not be expected to apply since the absorbance would be proportional to the square root of the amyl nitrite concentration. When an absorbance *versus* concentration plot is made for amyl nitrite, however, a straight line is obtained as shown in Fig. 2, indicating that the absorbing species cannot be in equilibrium as suggested. The absorbing species may result from an isomeric equilibrium reaction, in which case the applicability of Beer's law would indicate nothing.

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† Parke-Davis Fellow.

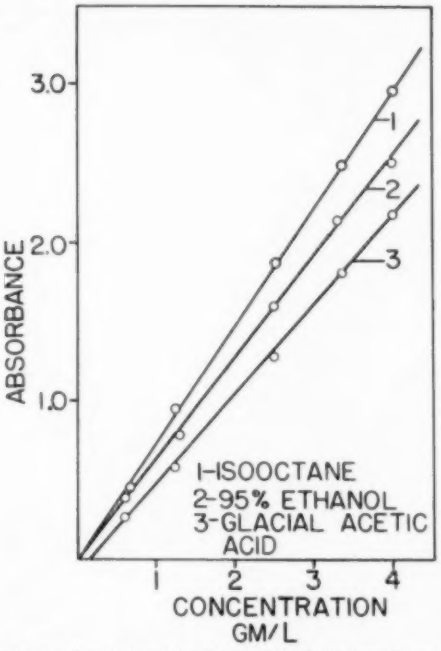

Fig. 2.—Beer's law plot of isoamyl nitrite in solvents indicated.

The possibility that the observed apparent low molar absorbance is due to a persistent, highly absorbing degradation product was studied experimentally. Assays were carried out on solutions of amyl nitrite in acetic acid which had been subjected to varying degrees of high temperature (104°) destruction. Determinations were made based on absorbances at 356.3 mμ and also by a modified method of Horsewell and Silverman (3), a colorimetric procedure involving the use of a copper-phenol complex. It was felt that it would be extremely unlikely that the rate of loss indicated by the two methods would correspond in the event that the ultraviolet absorbing species was other than the nitrite ester itself. The excellent correspondence of the two sets of results shown in Table II strongly supports the position that the absorption is due to amyl nitrite itself.

TABLE II.—ANALYTICAL RESULTS ON SAMPLES OF ISOAMYL NITRITE DEGRADED AT 104°

Time, Hr.	Decomposition by Method of Horsewell and Silverman, %	Decomposition by Absorbance at 356.3 mμ, %
0	0	0
2	15.7	15.4
3	26.5	25.0
4	36.1	37.6
7	65.2	65.2

In order to provide a further check on the possible effect of degradation products on the ultraviolet absorption pattern the spectrum of a five-year-old sample of amyl nitrite (pearl) which had undergone a substantial degree of breakdown was obtained as shown in Fig. 3. Analytical determinations were also made by the U. S. P. XIV (4) method and also by the modified Horsewell and Silverman method. The results of the assay by the three different analytical methods on this lot of amyl nitrite are shown in Table III.

TABLE III.—COMPARISON OF ANALYTICAL RESULTS OBTAINED WITH SEVERAL METHODS ON A FIVE-YEAR-OLD SAMPLE OF ISOAMYL NITRITE

Assay Procedure	Per Cent Remaining
U. S. P. XIV	76.6
Horsewell and Silverman	80.5
Absorption at 356.3 mμ	77.5

The data shown in the table strongly suggest that the several independent methods were at least measuring essentially the same quantity. The small discrepancies in the results are no more than would be normally expected from experimental variations. Despite this apparent agreement it should be pointed out that all of these methods may respond equally well to any nitrite ester or even to nitrous acid.

Ultraviolet absorption spectrum of the free acid in acetic acid-ethanol mixture appears to be identical with that of amyl nitrite in the same solvent. This would be expected since the rate of esterification and transesterification of nitrites is exceptionally high in the presence of acids.

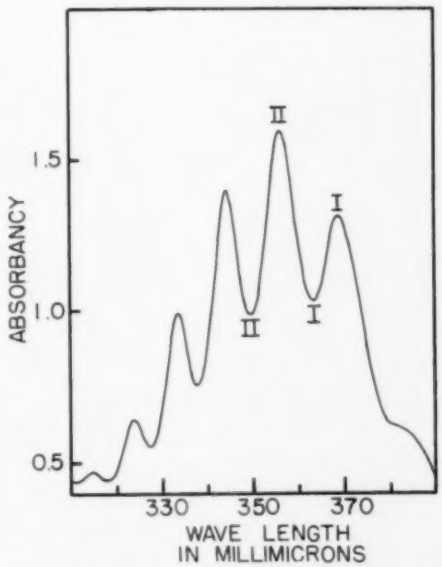

Fig. 3.—Near ultraviolet spectra of a five-year old sample of isoamyl nitrite in 95% ethyl alcohol. Solution contains 3 Gm. amyl nitrite per liter.

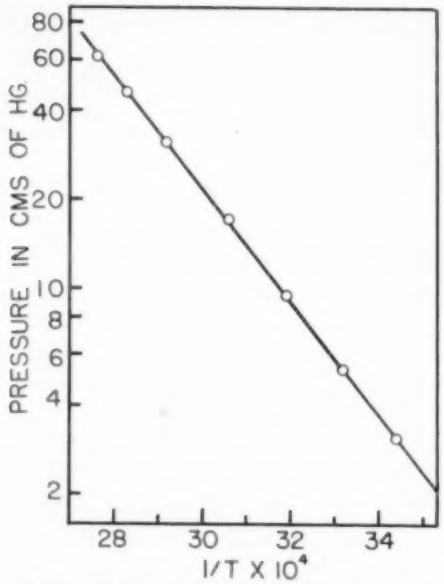

Fig. 4.—Vapor pressure of amyl nitrite as a function of the reciprocal of the temperature. Vapor pressure in cm. of Hg. Temperature in ° K.

It appears from the data given above that the observed ultraviolet absorption curves are apparently characteristic of the nitrite esters. The absorption pattern is unusually detailed for a spectrum obtained in solution and unexpectedly low.

VAPOR PRESSURE

The vapor pressure of amyl nitrite was measured as a function of the temperature by the Ramsey-Young method (5). The logarithms of the vapor pressure obtained are shown plotted against the reciprocal of the absolute temperature in Fig. 4. The equation which fits the overall straight line can be expressed as:

$$\log p = (-1916/T) + 7.082$$

where p is the vapor pressure in cm. Hg. The best fit equation in the 25° region differs but slightly:

$$\log p = (-1975/T) + 7.283$$

The exact values of the vapor pressure at several temperatures were interpolated from the experimental data and these are tabulated in Table IV. When the experimentally determined curve was extrapolated to 76 cm., the standard boiling point was found to be 96°. This value agrees favorably with the value of 97–99° reported in literature (6).

EXPERIMENTAL

Source of Amyl Nitrite.—The amyl nitrite used as reference standard was prepared according to the procedure of W. A. Noyes (7). Prior to its esterification, the isoamyl alcohol was fractionally distilled on a 50-plate Oldershaw fractionating column. The fraction taken for esterification distilled at 149–150°. After the ester was prepared it was distilled on the same column and a middle fraction taken. This fraction of amyl nitrite boiled

TABLE IV

Temperature, ° C.	Vapor Pressure, cm. Hg
0	1.13
10	2.02
20	3.50
25	4.54
30	5.84
40	9.44
50	14.8

in the range of 94–95°. The amyl nitrite was stored in amber-colored bottles which were kept refrigerated. Prior to being used the amyl nitrite was analyzed according to the procedure of the U. S. P. XIV and the analysis indicated that the amyl nitrite was 94.3% pure. A sample of decomposed amyl nitrite was obtained from ampuls prepared by Burroughs Wellcome & Co. lot No. 143 on November 26, 1951.

Modified Method of Horsewell and Silverman.—The method of Horsewell and Silverman was modified slightly. The reagent stock solution as described by the original workers was modified

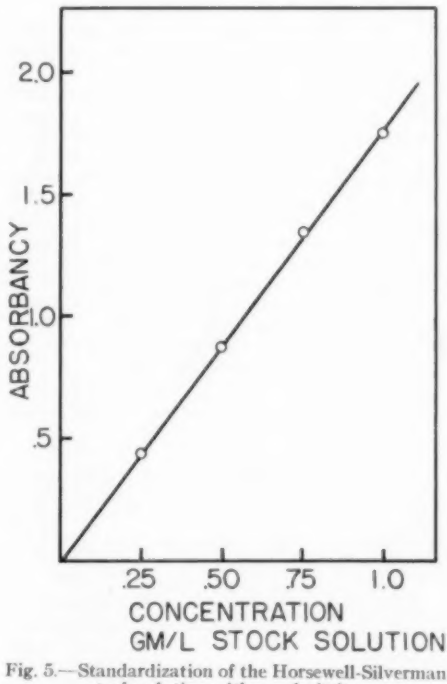

Fig. 5.—Standardization of the Horsewell-Silverman stock solution with amyl nitrite.

somewhat in order that the limitation on the amount of glacial acetic acid would not be exceeded. The stock solution used in this experiment was prepared containing: 5% phenol solution, 917 ml.; 2% hydrated copper sulfate solution, 33.0 ml.; glacial acetic acid, 45.6 ml. The stock solution was not allowed to stand more than eight hours before use. The validity of the modified method was calibrated experimentally on known samples as shown in Fig. 5.

Ultraviolet Absorption.—All runs were made on a Cary Recording Spectrophotometer.

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Catecholase Activity in Tissue Cultures of *Claviceps litoralis**

By S. K. SIM† and H. W. YOUNGKEN, Jr.‡

An acetone powder catecholase preparation was obtained from the pigmented tissues of *Claviceps litoralis*. Determined by a monometric method, the catecholase activity was found to be low in younger, less pigmented tissues, and higher in older, more pigmented tissues. It reached a maximum shortly after the peak of the growth period and declined slightly thereafter. The maximum catecholase activity occurred in twenty-eight to thirty-five days of growth. The catecholase activity of the powder preparation was found to be proportional to the concentration of the powder enzyme preparation, and was inhibited by sodium diethyldithiocarbamate and by potassium cyanide. The powder preparation of young tissues showed some ability to oxidize tyrosine and dopa, but at a very low rate in comparison with catechol oxidation. There was no oxidation of tyrosine and dopa by enzyme preparations from the older tissues which possessed high catecholase activity.

THE ABILITY of *Claviceps litoralis* to produce a significant amount of a purplish-black pigment in artificial culture has been reported (1). However, no alkaloid was found to be biosynthesized during the pigmentation in the mycelial tissues of the fungus or in the culture liquid. On the other hand, Loo and Lewis (2) have reported that the alkaloid formation in *Claviceps purpurea* grown on rye plants began at about the same time as the production of pigment in that species, thus implicating an association between pigmentation and alkaloid formation. Several proposals (3–5), though not experimentally confirmed, indicate that the metabolism of tyrosine, which includes oxidative enzyme activity, might play an important role in the initial steps of the biosynthesis of the indole compounds and the ergot alkaloids. Furthermore, Dawson, Lerner, and others (6–10) have shown that tyrosine and its related metabolites are intermediates in melanin and melanin-like pigmentation in plants and anima's.

It would seem that if tyrosine or related "precursor" compounds, (i. e., hydroxyphenylalanine) are involved directly or indirectly in the biosynthesis of Claviceps pigmentation, or its alkaloids, in artificial culture, one should be able to demonstrate the presence of a tyrosinase or similar phenol oxidase reaction in the fungus growth. Catecholase activity is often employed to demonstrate these reactions. This report describes the ability of an enzyme preparation from *Claviceps litoralis* Kawatani to show marked catecholase activity and only slight oxidation of L-tyrosine or dihydroxyphenylalanine.

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Abstracted from a thesis submitted to the Graduate School of the University of Washington by S. K. Sim in partial fulfillment of the requirements for the degree of Doctor of Philosophy. This thesis won the *Edwin Leigh Newcomb Award* in Pharmacognosy for 1956. The study has been supported in part by the State of Washington Funds for Medical and Biological Research.

EXPERIMENTAL

A strain of *Claviceps litoralis* Kawatani previously developed in these laboratories (1) was selected for the study since this was found to produce excellent purplish-black pigmentation under artificial culture conditions. A homogenized inoculum of this strain was prepared as follows: A small piece of surface mycelium from a stock culture was placed under sterile conditions into 500-ml. Erlenmeyer flasks with 100 ml. of sterilized liquid medium of the following formula:

Potassium dihydrogen phosphate	2.50 Gm.
Magnesium sulfate (heptahydrate)	1.25 Gm.
Peptone (Difco)	1.25 Gm.
Yeast extract (Difco)	12.50 Gm.
Mannitol	20.00 Gm.
Water, sufficient to make	1.00 liter

Flasks were shaken continuously on a mechanical shaker at 200 excursions per minute for fourteen days whereupon the mycelial growth was transferred under aseptic conditions into a sterilized stainless steel Waring blendor cup. The material was then homogenized for about one minute to produce a finely ground tissue suspension. Two milliliters of this tissue homogenate (about 2 mg. dry weight) was then used as a standard inoculum for developing experimental growth cultures of the Claviceps.

Each series of experimental growth cultures was inoculated with 2 ml. of the standard inoculum per flask and the mycelial growth was collected from 6 or 7 flasks for enzyme study after 9, 16, 20, 28, 35, 42, and 49 days of growth. Tissue was harvested by suction filtration using sintered glass crucibles. In each case it was thoroughly washed with distilled water. Washed tissues were then lyophilized and dry weights determined. The volume of culture liquid was also measured and its *p*H determined. Results are shown in Table II.

Enzyme Preparations.—Numerous attempts were made to prepare the most active enzyme preparation for measuring an oxidative system in the Claviceps growth. For this purpose tissue materials from several flasks were separated from the culture liquid by centrifuging at 2000 g. for five minutes, the supernatant liquid being decanted off. The tissues were then washed with distilled water until the wash water was colorless. After the last washing, the wet tissues were filtered by suction on a Büchner funnel, and then lightly pressed by hand between filter papers, and the fresh weight determined. The tissues were then used for preparing an acetone powder enzyme preparation.

In preliminary experiments with *C. litoralis* cultures it was found that pieces of tissues which were washed but not ground or powdered, catalyzed catechol oxidation. This was observed by manometric measurements of the oxygen uptake. An acetone powder was then prepared from these harvested and washed tissues. This acetone powder preparation was also found to oxidize catechol. Attempts were then made to obtain a soluble enzyme preparation. The harvested and washed tissues were homogenized in *M*/15 phosphate buffer, *p*H 6.2, in a chilled motor-operated glass homogenizer. The resulting homogenate showed catecholase activity, but as it also showed considerable endogenous oxygen uptake, the increased oxygen uptake in the presence of catechol was not sharply defined. When this homogenate was centrifuged the supernatant liquid showed no activity with catechol; the centrifuged tissue particles, however, from the homogenate, after resuspension in buffer, retained almost all of the activity of the whole homogenate (Fig. 1). Apparently,

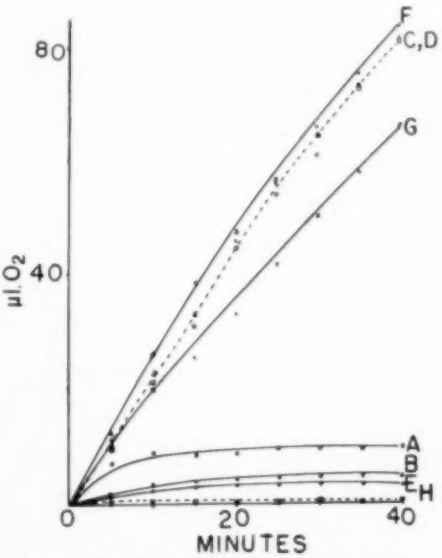

Fig. 1.—Catecholase activity of Claviceps tissue homogenate. A and B—homogenate; C and D—homogenate + catechol; E—resuspended residue from centrifuged homogenate; F and G—same as E but in presence of catechol; H—supernatant from centrifuged homogenate, with and without catechol. Catechol 0.002 *M* in buffer *p*H 6.2 at 30°.

the homogenizing procedure failed to disrupt the cell walls and release the enzymes. Grinding procedures were then employed as follows: By means of a chilled mortar and pestle, one part fresh weight of harvested and washed tissues, which showed catecholase activity before grinding, was ground for five minutes with each of one-half part, one part, and three parts of washed sand. Carborundum was also used in a similar way. These mixtures were then centrifuged. The supernatant liquid in each case was entirely inactive when examined for enzyme activity by manometric techniques. In each case the tissue was ground in chilled *M*/15 phosphate buffer, *p*H 6.2 and 7, and the grinding was carried out immediately prior to use.

Another attempt was made to obtain a cell-free enzymatically active extract by disintegrating the tissue materials in a Mickel disintegrator. This was done in a cold room for fifteen minutes with 1 Gm. fresh weight of tissue to 5 Gm. of glass beads. The resultant supernatant liquid after centrifuging was again inactive when tested for catecholase activity. Disintegration of the tissue in a sonic vibrator in a cold room for thirty minutes was like-

wise not effective in producing a soluble enzyme preparation.

Acetone Powder Enzyme Preparation.—Since the acetone powder preparation of the mycelial tissues consistently showed good catecholase activity in preliminary experiments, this preparation was adopted for subsequent use. The procedure was adapted from the methods of James, *et. al.* (11), for *Atropa belladonna* leaves and of LaDu and Greenberg (12) for rat liver preparations with slight modification. After the last washing of the tissues in the harvesting procedure, the water adhering to the tissues was further removed by suction filtration with a Büchner funnel. This moist mass was then lightly pressed by hand between filter papers, and its weight determined as the fresh weight. It was then ground with chilled acetone (5 ml. of acetone per Gm. of tissue fresh weight) in a chilled Waring blendor at low speed for two minutes, and then filtered through filter paper on a chilled Büchner funnel. Before it was completely sucked dry, the powder on the funnel was transferred back to the blendor cup and ground for two minutes with a further quantity of acetone and again filtered through the Büchner funnel. This was then washed twice with acetone. The powder was then sucked dry on the funnel, and lyophilized for three hours. The dry powder was then ground in a mortar and stored in a desiccator for fifteen hours over calcium chloride. One hundred 20-mg. aliquots of this powder were used in each Warburg reaction vessel for enzyme activity studies.

Estimation of Catecholase Activity.—The powdered enzyme preparation was placed in the main compartment of the Warburg reaction vessel with $M/15$ phosphate buffer at pH 6.2. The substrate was placed in one side arm, and an enzyme inhibitor (when used) in the other side arm. The total volume of fluid in the main compartment and in the side arms was 3 ml. The center well contained 0.2 ml. of 20% potassium hydroxide with a filter paper strip. The contents of the side arms were tipped in after the equilibration of the vessels and their contents maintained in a constant temperature bath at 30° for fifteen minutes. The vessels were shaken at 100 oscillations per minute. The substrates were added from the side arms at zero time after the initial equilibration as 0.2 ml. of 2×10^{-2} M catechol, 0.3 ml. of 1.5×10^{-2} M L-tyrosine in 1.5×10^{-2} M NaOH, or 1 ml. of 6×10^{-3} M L-dopa. This generally gave a final concentration in the reaction mixture of 2×10^{-3} M for catechol, 1.5×10^{-3} M for tyrosine, or 2×10^{-3} M for dopa. Sodium diethyldithiocarbamate (dieca), when used as inhibitor, had a concentration in the reaction mixture of 1.5×10^{-4} M and 1×10^{-3} M. Manometric readings were taken at five-minute intervals for the first sixty minutes, and thereafter at ten-minute intervals for an additional sixty minutes to one hundred twenty minutes. Control vessels were always run for each experiment with the enzyme preparation in buffer and with buffer tipped in from the side arm instead of substrate.

Catechol with a final concentration of 2×10^{-2} M was used as the substrate for all cases in which the catecholase activities of cultured tissues from different periods of growth were compared. In each experiment, with enzyme preparations from the same batch of harvested tissues, 3 to 6 replicate vessels were run with the same substrate. The oxygen uptake per 120 mg. enzyme preparation was calculated and plotted against time for each vessel. The maximum rate of oxygen uptake, as µl. of O_2 per hour per 120 mg. enzyme preparation, was determined from the portion of the graph the slope of which showed a maximum rate for that graph. Such a maximum rate was taken only when the maximum rate was sustained, (i. e., that portion of the graph was linear) for twenty minutes or more. In most cases this occurred as the portion of the graph for the first thirty or forty minutes after addition of substrate. In a few cases, there was a five-minute lag period at the beginning. These maximum rates from the replicates of the same experiment, as well as those from the replicates of other experiments with enzyme preparations of cultures of the same age, were grouped together for computing the average maximum rate of O_2 uptake. Similarly, the replicates of control vessels from these same experiments were grouped together, and their average rates of O_2 uptake were computed. The range of deviations among such replicates may be seen from the standard deviation of the mean calculated for each group. The excess of the mean maximum rate of O_2 uptake for experimental samples (with substrate) over and above that of the control samples constituted the catecholase activity of the enzyme preparations from cultures of that particular age (period of growth). This activity was calculated both on the basis of per 120 mg. enzyme preparation and on the basis of per mg. of nitrogen in the enzyme preparation.

Nitrogen Determination.—The nitrogen content of the acetone powder enzyme preparations was determined as total nitrogen by a modification of the methods of Ballentine and Gregg (13) and of Pepkowitz and Shive (14), by means of a micro-Kjeldahl apparatus. A mixture of sulfuric acid, selenium oxychloride, and perchloric acid was used for the digestion. The digest was distilled into a standard potassium bi-iodate solution, which, after addition of KI at the completion of distillation, was subsequently titrated with standard sodium thiosulfate solution to a starch end point.

RESULTS

Catecholase Activities of Claviceps Cultures of Different Ages.—Typical means of O_2 uptake, at various time intervals, by the powder enzyme preparations in the presence of catechol (2×10^{-2} M) and corrected for endogenous respiration is shown in Fig. 2. The crude enzyme preparations in buffer without substrate (controls) manifested a certain amount of oxygen consumption. This endogenous O_2 uptake was greater in actual quantity as well as in rate in the enzyme preparations from younger cultures than in those from older cultures. For instance, those from sixteen-day-old cultures consumed 22 µl. of O_2 in the first forty minutes and 49 µl. of oxygen in one hundred minutes, whereas the enzyme preparations from the forty-two-day-old cultures consumed 8.5 µl. and 16 µl. of oxygen, respectively, in the above periods. But in all cases, the enzyme preparations showed a considerable increase of oxygen uptake in the presence of catechol. For example, enzyme preparations from sixteen-day-

TABLE I.—CATECHOLASE ACTIVITIES OF CRUDE ENZYME PREPARATIONS FROM CLAVICEPS CULTURES OF DIFFERENT AGES

Age of Cultures (days)	Mean Maximum Rate of O_2 Uptake (μl./120 mg./hr.) ± S. D.[a] Without Substrate (a)	With Substrate (b)	Mean Maximum Rate of O_2 Uptake (μl./mg. N/hr.) Without Substrate (c)	With Substrate (d)	Catecholase Activity (e) As μl. O_2/120 mg./hr. (b) − (a)	(f) As μl. O_2/mg. N/hr. (d) − (c)	N Content of Enzyme Preparation, %
16	37.7 ± 2.5	80.7 ± 1.6	5.6	12.1	43.0	6.5	5.58
20	23.3 ± 0.1	129.5 ± 7.2	3.6	19.8	106.2	16.2	5.46
28	29.4 ± 2.1	219.1 ± 9.4	4.5	33.3	189.7	28.8	5.48
35	10.4 ± 1.4	220.1 ± 7.0	1.4	29.1	209.7	27.7	6.31
42	18.3 ± 1.0	152.9 ± 2.6	2.4	20.4	134.6	18.0	6.26
49	15.6[b]	190.8[b]	2.1	25.4	175.2	23.3	6.25

[a] S. D. = standard deviation of the mean = $\pm\sqrt{\Sigma d^2/n/(n-1)}$.
[b] S. D. was not calculated as only a small number of samples were run.

TABLE II.—GROWTH DATA OF *Claviceps litoralis* CULTURES

Period of growth, days	9	16	20	28	35	42	49
No. of flasks	5	6	6	6	6	6	6
Mean tissue dry wt. per flask (mg.)	99.0	649.0	1000.9	1179.5	941.7	686.1	544.8
Standard deviation of the mean (± mg.)	±4.8	±13.7	±21.8	±30.7	±14.9	±19.2	±12.0
*p*H of culture liquid at harvest	5.95	5.50	5.72	6.30	6.85	6.70	6.80
Average volume of culture liquid per flask at harvest (ml.)	93	83	75	72	70	70	68

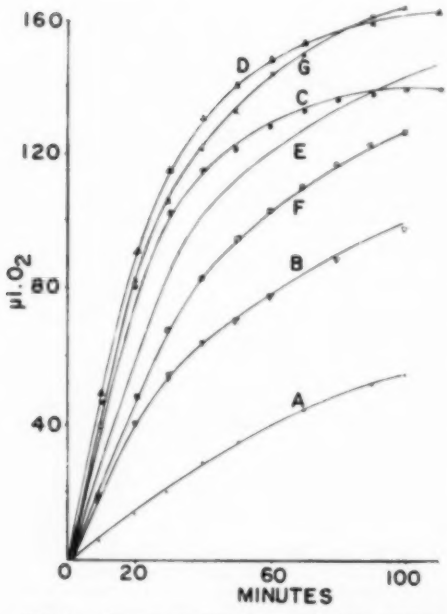

Fig. 2.—Oxygen uptake by catecholase preparations from Claviceps cultures of different ages (corrected for endogenous respiration). A—16 days; B—20 days; C—28 days; D and E—35 days; F—42 days; G—49 days. Substrate; catechol 0.002 *M* at *p*H 6.2 and 30°.

old cultures and forty-two-day cultures consumed 50 μl. and 92 μl. of oxygen, respectively, in the first forty minutes in the presence of catechol.

The mean maximum rates expressed as μl. of oxygen per 120 mg. powder per hour are listed in columns (*a*) and (*b*) in Table I. The absolute quantities of oxygen uptake differed among different individual samples of the same experiment or of different experiments using preparations from cultures of the same age. But when plotted out, the rates of oxygen uptake of samples prepared from cultures of the same age did not differ significantly.

For the enzyme preparations from the harvested tissues of any age, the excess of the mean maximum rate of O_2 uptake in the presence of catechol when compared with the mean maximum rate of O_2 uptake in the absence of catechol, constituted the catecholase activity. These are shown in columns (*e*) and (*f*) of Table I.

A comparison of the different catecholase activities of the powder preparations from cultures of different ages is represented graphically in Fig. 3 together with a comparison of the tissue dry weights. See Table II for other pertinent growth data.

From Fig. 3 it will be noted that the catecholase activity of powder preparations made from nine to ten-day-old or younger cultures was practically nil. This catecholase activity began to increase with the age of the culture until a maximum was reached at thirty-five days if calculated on the basis of per unit weight of the crude enzyme preparation, or at twenty-eight days if calculated on the basis of per mg. of nitrogen. On either basis, the maximum of the catecholase activity was close to the peak of the dry weight. The dry weight also began to decline

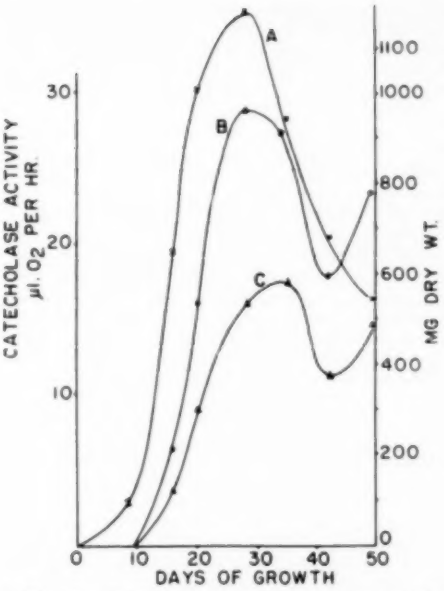

Fig. 3.—Catecholase activity and dry weight changes in Claviceps cultures. A—tissue dry wt. per flask; B—catecholase activity as µl. O_2 per mg. N per hour; C—catecholase activity as µl. O_2 per 10 mg. powder per hour.

after the maximum at about twenty-eight days, apparently due to autolysis of the fungal tissues after exhaustion of essential nutrient materials in the medium or accumulation of toxic products. Pigmentation of the cultured tissues also began to occur about ten to twelve days after inoculation, and the whole culture became progressively more pigmented as time went on. The tissues in the whole flask became entirely pigmented in about four to five weeks after inoculation. As noted above, maximum catecholase activity occurred at about this time.

Catechol Oxidation by Different Concentrations of the Powder Preparation.—Efforts were made to determine whether the catecholase activity would vary in proportion to variations of the crude enzyme powder preparation. Powders prepared from

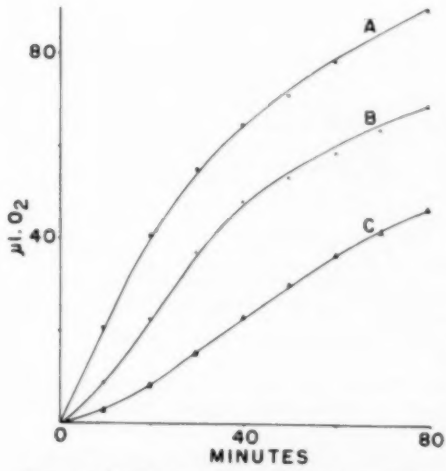

Fig. 4.—Oxygen uptake by different concentrations of Claviceps catecholase preparation. Powder preparation per reaction vessel 120 mg. (A), 80 mg. (B), and 40 mg. (C), in 3 ml. reaction mixture. O_2 uptake corrected for endogenous respiration; substrate: catechol 0.002 *M* at *p*H 6.2 and 30°.

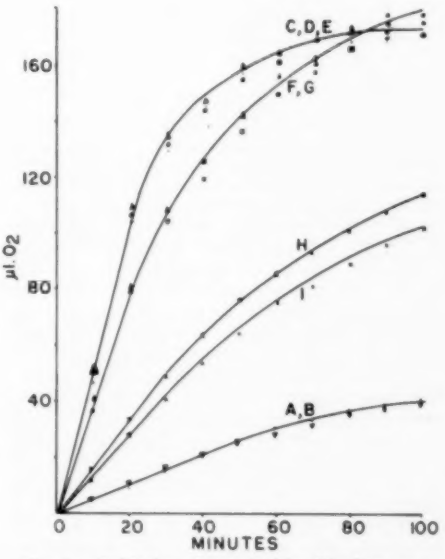

Fig. 5.—Oxidation of catechol by Claviceps powder enzyme preparation and its inhibition by diethyldithiocarbamate (dieca). A and B—powder in buffer; C, D, E, F, G—powder in buffer + catechol; H and I—powder in buffer + catechol + dieca. Powder preparation from twenty-eight-day cultures; buffer *p*H 6.2; catechol 0.002 *M*; dieca 1.5×10^{-4} *M*.

twenty-day cultures were used in concentrations of 40, 80, and 120 mg. in 3 ml. of reaction mixture. The substrate was 2×10^{-3} *M* catechol in *M*/15 phosphate buffer at *p*H 6.2 in all cases. The results are shown in Fig. 4. Each point in Fig. 4 represents the mean of three replicates.

Inhibitors.—The catecholase or tyrosinase inhibitors, sodium diethyldithiocarbamate (dieca) and potassium cyanide, were employed for testing their inhibitory effect on the enzyme activity of the powder preparations. Results are shown in Figs. 5 and 6. It may be seen that dieca at 1.5×10^{-4} *M* brought about approximately 50% inhibition. When dieca was used in concentration of 8×10^{-4} *M* nearly 100% inhibition occurred. Potassium cyanide was used in concentrations of 1×10^{-3} *M* and 2×10^{-3} *M*. In Fig. 6, it is apparent that the

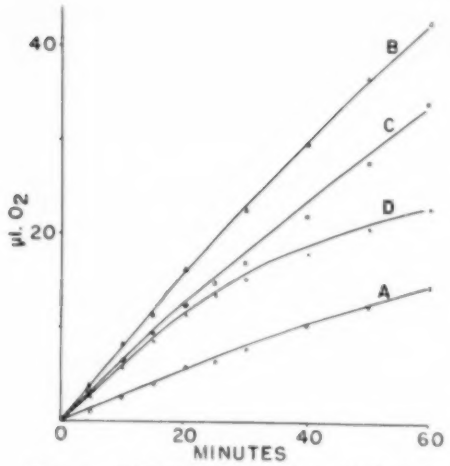

Fig. 6.—Inhibition of catechol oxidation by cyanide. A—Claviceps preparation in buffer; B—Claviceps preparation in buffer + catechol; C—Claviceps preparation in buffer + catechol + KCN 0.001 *M*; D—Claviceps preparation in buffer + catechol + KCN 0.002 *M*. Catechol 0.002 *M* at *p*H 6.2 and 30°.

crude enzyme powder preparation was fairly resistant to cyanide inhibition. A 2×10^{-3} *M* concentration brought about only 50% inhibition. James, *et al.* (11), reported a 50% inhibition by 1×10^{-3} *M* cyanide in a catecholase powder preparation from *Atropa belladonna* leaves.

Oxidation of Tyrosine and Dopa.—The powder preparations from the sixteen, twenty, twenty-eight, and thirty-five-day cultures were tested for their ability to oxidize L-tyrosine (1.5×10^{-3} *M*) and L-dopa (2×10^{-3} *M*) at *p*H 6.2 when the latter compounds were added to enzyme preparations. Powder preparations from two different lots of sixteen-day-old cultures showed a slight oxidation of L-tyrosine in comparison with that for catechol oxidation by the same powder preparation. The powder preparation from 20-day cultures also showed a slight oxidation of L-dopa. Twenty-eight and thirty-five-day cultures showed no ability to oxidize either L-tyrosine or L-dopa. It was noted, on the other hand, that the powder preparations from older cultures showed high catecholase activity.

DISCUSSION

The results show that the cultured tissues of *Claviceps litoralis* contained an enzyme with considerable catecholase activity. The most active preparations, for example, those from the twenty-eight-day cultures, showed an oxygen uptake rate (corrected for endogenous oxygen uptake) of 29 μl. per mg. nitrogen per hour, with catechol as the substrate.

The enzyme preparations from the older, more pigmented cultures (twenty-eight and thirty five-day cultures) had higher catecholase activities than those from the younger and less pigmented tissues. It is not clear what mechanism was responsible for these variations of catecholase activity with age of the culture. Increase of enzyme activity could perhaps be due to conversion of nonenzyme protein to enzyme protein, or it might be due to release of active form of the enzyme from some protein complex which contained an inactive precursor of the enzyme. It might be due to release of some activator in the process of tissue autolysis, or to the destruction of inhibitors. However, these factors were not investigated in the present study. It is interesting to note that somewhat analogous variations in tyrosinase activity of the enzyme preparations obtained from a pigmented strain of *Glomerella cingulata* (an ascomycete) were reported by Sussman and Markert (6).

The ability of this Claviceps catecholase preparation to specifically oxidize tyrosine and dopa was very low, and this occurred only with preparations from the younger cultures and not at all with the older cultures. The oxidation of catechol by this same preparation was also inhibited by cyanide and by diethyldithiocarbamate (dieca). Thus, the enzyme preparation appears to have several properties in common with polyphenol oxidase and tyrosinase preparations described for various animal and plant sources (6, 7, 8, 9, 11).

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The Pharmacological Action of 1-*o*-Chlorophenyl-2-Isopropylaminoethanol (Isoprophenamine[1]) A Bronchodilator*

By C. E. POWELL, W. R. GIBSON, and E. E. SWANSON

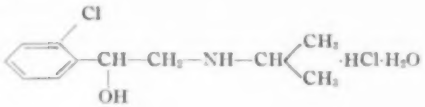
Pharmacological studies show a synthetic compound, 1-*o*-chlorophenyl-2-isopropylaminoethanol,[2] to possess bronchodilator activity. The pilocarpine-constricted bronchi of decerebrate and pithed dogs are rapidly relieved by the compound. In anesthetized dogs, the compound has a depressor action with increased heart rate. Tests on guinea pig tracheal chains and isolated perfused lungs of rabbits also show the compound to have bronchodilator action against pilocarpine-induced constriction. Toxicological studies indicate the compound has a low order of toxicity. Diets containing 0.1% and 0.5% of the compound do not affect the weight gain of rats. Larger amounts of the drug retard the weight gain. Isolated smooth muscle tests show the compound to have weak antihistaminic and antispasmodic activity.

SINCE THE PUBLICATION of reports (1–3) that ephedrine and related compounds have a bronchodilator action, continuous search for a synthetic substitute having this property nas been conducted. The ultimate purpose of this search is the synthesis of a substance that has a bronchodilator action of longer duration than epinephrine and is more specific in its effect than ephedrine.

Recently a series of compounds related to epinephrine were synthesized by our organic chemistry division. One of the compounds, 1-*o*-chlorophenyl-2-isopropylaminoethanol, appeared to have a marked bronchodilating action. The present investigation deals with pharmacological and toxicological studies of this compound.

EXPERIMENTAL

General Properties.—As a hydrochloride hydrate salt 1-*o*-chlorophenyl-2-isopropylaminoethanol is a white, crystalline compound melting at 161–162° (corrected). It is easily soluble in distilled water at room temperature. The structural formula is as follows:



It will be noted that the compound, hereafter referred to as compound 20025, bears an ortho-substituted chlorine in the phenyl ring, and further, does not have the phenolic hydroxy group.

Methods. — For its bronchodilator action, compound 20025 was studied in 8 decerebrate and pithed dogs prepared by the methods of Elliott (4) and Jackson (5). Additional preparation of the animals included treatment by vein with 0.5 mg./Kg. of ergotoxine ethanesulphonate followed by 1 mg. of pilocarpine HCl which immediately constricted the bronchi. Epinephrine was given by vein in order to block the bronchoconstrictor action of pilocarpine temporarily and to prevent asphyxiation of the dogs.

The blood pressure and respiration effects of compounds 20025 were recorded in 4 dogs under anesthesia. Three of the animals were anesthetized by intravenous injections of 150 mg./Kg. of sodium phenobarbital and one dog by 0.6 cc./Kg. of Dial-Urethane. Electrocardiograms were obtained on one dog under sodium phenobarbital anesthesia that received 2 mg./Kg. of compound 20025 by vein.

Oral and intravenous toxicity studies of compound 20025 were conducted on 60 starved albino mice and 25 starved rats. The $LD_{50} \pm$ S. E. was calculated by the method of Bliss (6). By a procedure previously described (7) the subacute toxicity was studied in rats and dogs. Four groups of 5 female rats each were fed a diet containing 0.05% and 0.5% of compound 20025. One group of 5 female rats was fed the untreated diet for control observations. Six dogs, divided into two groups of three each, were given daily doses of 25 mg./Kg. and 50 mg./Kg. of compound 20025 by capsule. During the period of treatment, blood and urine analyses were made. After 30 days of treatment the animals were sacrificed and submitted for necropsy.

In vitro studies were conducted on the tracheal chain of guinea pigs according to the method of Castillo and DeBeer (8), and the rabbit lung method of Sollmann and Von Oettingen (9). In both preparations the tissues were contracted with 1 mg. of pilocarpine HCl and the releasing action of compound 20025 was compared with that of epinephrine. Altogether 6 tracheal chain preparations and 4 lung preparations were used.

The smooth muscle action of compound 20025 was studied on the isolated ileums and uteri from 3 rabbits and 4 guinea pigs. In each case the

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[1] Generic name.

[2] Originally identified as compound 20025.

isolated organ was suspended in a 100-cc. muscle chamber containing Tyrode's solution maintained at 37.5°. Kymograph tracings were taken with a gravity writing lever. Final test dilutions of compound 20025 varied from 1–5,000 to 1–400,000. The antispasmodic and antihistaminic action of compound 20025 was evaluated against methacholine and histamine spasm of the isolated ileum of guinea pigs.

RESULTS AND DISCUSSION

Intravenous doses of compound 20025 promptly dilated the pilocarpine-constricted bronchi of decerebrate and pithed dogs. Doses of 1.75 mg./Kg., 2 mg./Kg., and 5 mg./Kg. were as rapid in releasing the constriction as 0.25–0.5 μg./Kg. of epinephrine, and as effective as ephedrine in sustaining the bronchodilator action. Figure 1 shows a typical response obtained with 1.75 mg./Kg. of the compound. In all cases the blood pressure responded with a slight, brief fall followed by a slight, sustained rise. Smaller doses (1.5 mg./Kg. and 1 mg./Kg.) were slower to bring about the maximum release of pilocarpine-induced constriction; however, the dilator response in all tests was more prolonged than that observed with epinephrine.

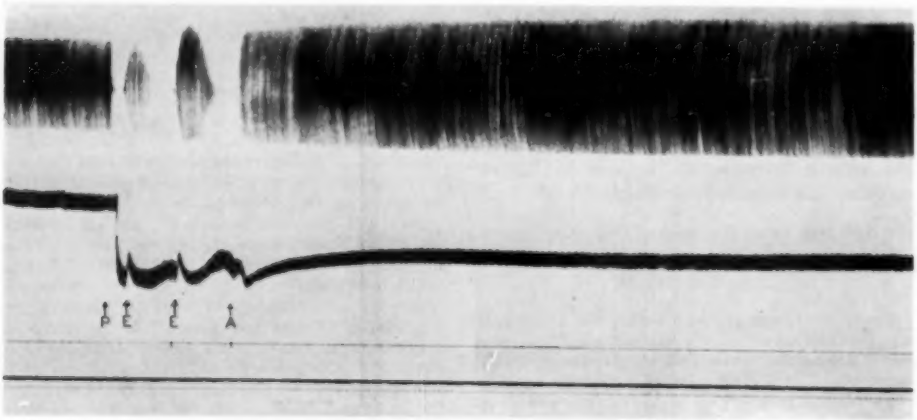

Fig. 1.—Action of compound 20025 in a decerebrate and pithed dog, male, 7.2 Kg., vagi cut. Tracings from top to bottom are: Bronchi, blood pressure, signal marker and base line. Time in minute and 10-second intervals. P: bronchial constriction and depressor action caused by 0.15 mg./Kg. of pilocarpine. E: epinephrine, 0.25 μg./Kg. Second E: epinephrine 0.50 μg./Kg. A: 20025, 1.75 mg./Kg. Note the sustained response to the compound.

In dogs anesthetized with sodium phenobarbital, compound 20025 in intravenous doses of 5 mg./Kg. increased the respiration rate from 25 times per minute to 32 times per minute. The mean arterial pressure was lowered an average of 24 mm. Hg for more than one hour. The heart rate was increased an average of 84 beats per minute. Under Dial-Urethane anesthesia, a 2 mg./Kg. dose of compound 20025 lowered the mean arterial pressure 15 mm. Hg and increased the heart rate by 40 beats per minute. The respiration rate was increased from 22 to 25 times per minute. In one dog under sodium phenobarbital anesthesia, a 2 mg./Kg. dose of the compound had no observable effect on the cardiac action except that the heart rate increased from 84 to 120 beats per min. The electrocardiogram showed no change, and no arrhythmias were noted.

The tracheal chain showed compound 20025 to have a relaxing action against pilocarpine-induced spasm. From an average of six tests it was estimated that the potency of 1 mg. of the compound was equivalent to that of 9 μg. of epinephrine in releasing pilocarpine-induced spasm. On the isolated rabbit lung, compound 20025 was found to have a more potent action. From an average of 4 tests it was estimated to be 47% as active as epinephrine in releasing pilocarpine constriction. The synthetic compound, therefore, has a more potent action on the bronchial tree than on the tracheal muscle structure.

Acute toxicity studies by vein in starved albino mice showed compound 20025 to have an $LD_{50} \pm$ S. E. of 61.86 ± 4.76 mg./Kg. Fatal doses produced immediate convulsions followed by death of the animals in from one to five minutes. In starved albino mice the oral $LD_{50} \pm$ S. E. was calculated to be 298.4 ± 36.6, and in starved rats it was estimated to be 600 mg./Kg. In all toxicity tests, death of the animals occurred soon after administration of the drug.

The results of subacute toxicity tests in rats are shown in Table I.

Animals in the groups receiving 0.05% and 0.10% of compound 20025 in the diet over a 30-day period had approximately the same weight gain as the control. However, animals on a diet that contained 0.25% and 0.50% of the drug showed definite retardation of weight gain during the 30-day treat-

ment. Gross and microscopic postmortem examination showed all rats but one to be normal. This one animal had a unilateral hydronephrosis, a condition observed frequently in rats and obviously not attributable to the drug treatment. Terminal blood counts appeared to be normal.

TABLE I

Drug in Diet, %	Av. Daily Food Intake, Gm.	Av. Daily Drug Intake, Mg.	Increase in Body Weight, Gm.
Control	13.1	..	79.0
0.05	12.8	6.4	71.4
0.10	13.9	13.9	77.8
0.25	12.7	31.8	45.0
0.50	13.3	66.5	48.2

The weight of dogs receiving 25 mg./Kg. and 50 mg./Kg., respectively, by mouth was not significantly changed after 30 days of treatment. Only one dog vomited occasionally after a 50 mg./Kg. dose. All dogs were sacrificed and submitted for necropsy at the end of experimentation. Gross and microscopic findings of the heart, lungs, liver, pancreas, spleen, stomach, intestines, kidneys, thyroid, and adrenal glands were normal. One dog receiving doses of 50 mg./Kg. showed mild pyelonephritis. As in the rats, terminal blood counts appeared to be normal.

When tested on unstimulated, rhythmically contracting isolated ileum preparations of rabbits, compound 20025 reduced the amplitude of contractions and relaxed the tone with dilutions of 1:400,000 to 1:1,000,000. Dilutions up to 1:5,000 had no effect on the isolated uteri of guinea pigs and rabbits. The isolated ileum of guinea pigs was relaxed with dilutions of 1:40,000 to 1:200,000 of compound 20025. Also, on isolated guinea pig ileum, compound 20025 had weak antihistaminic and antispasmodic action. Dilutions of 1:40,000 were required to release spasms induced by histamine or methacholine.

Thus compound 20025 appears to exert selective action directly on the bronchial tree as shown in the decerebrate and pithed dog preparations. Also, the drug exhibits a pronounced relaxant effect on the isolated lungs of rabbits. In blood pressure experiments on anesthetized dogs, a marked tachycardia occurs with large doses of the compound; however, with small effective doses the increased heart rate is only slightly above normal.

SUMMARY

In a series of pharmacological and toxicological studies, compound 20025 (1-*o*-chlorophenyl-2-isopropylaminoethanol) was found to possess bronchodilator activity

1. In decerebrate and pithed dogs compound 20025 rapidly relaxed pilocarpine-constricted bronchi and maintained this action longer than epinephrine. Anesthetized dogs responded to the drug with slight lowering of blood pressure and increased heart rate, with no changes in the electrocardiogram.

2. In the perfused lung of rabbits compound 20025 had a bronchodilating action against pilocarpine constriction, being 47% as active as epinephrine. The tracheal chain of guinea pigs showed that 1 mg. of the compound equaled 9 μg. of epinephrine in releasing pilocarpine spasm.

3. In mice the intravenous $LD_{50} \pm$ S. E. was 61.86 ± 4.76 mg./Kg. Oral toxicities in mice and rats were lower.

4. In subacute toxic tests in rats, 0.05% and 0.1% of the drug in diets did not affect weight gain. Larger amounts of the drug in the diet (0.25% and 0.5%) retarded the weight gain. Terminal blood counts were normal, and no abnormal postmortem changes were observed.

5. Isolated smooth muscle tests show that 20025 has a weak antihistaminic and antispasmodic action.

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Drug Addiction I. Addiction by Escape Training*

By JOHN R. NICHOLS,† CECIL P. HEADLEE,‡ and HAROLD W. COPPOCK§

Withdrawal has been shown to be a motivating state which may be used to promote learning a new action, if this new action reduces the level of withdrawal. The withdrawal syndrome may be considered a noxious stimulus to which the animal learns an action which reduces the severity of the noxious stimulus. This form of learning is called escape training and is a form of conditioned response. Relapse by animals was demonstrated and the importance of the confirmation of the escape training hypothesis was discussed.

DURING THE PAST decade there has been an ever increasing awareness of the many problems related to drug addiction. Legislation to control narcotic traffic, and more recently, the treatment of addicts, has accomplished much to alleviate many of the problems. Little has been accomplished however toward solving the problems as to the more basic and underlying principles causing addiction.

The authors have previously reported some preliminary results of a laboratory method of detecting drug addiction (1). Among other things, the results of this investigation revealed that the learning of rats to prefer a given head position was dependent upon a restoration of homeostatic equilibrium which accompanied a particular head position. This then implies that withdrawal may be a motivating state, just as hunger is a motivating state. It is a well established principle that animals tend to learn those actions which result in decreasing their motivational level (2, 3). Thus, if both hunger and withdrawal may be considered as motivating states, then the animal should learn those actions which reduce the motivational level in either case. This is known to be true in the case of the hungry animal, but is it true also for the animal suffering withdrawal symptoms?

If so, more basic results of this previously reported experiment on addiction may be interpreted in these terms. There was (*a*) a *motivated* animal which could by (*b*) its *actions* (*c*) *reduce* the motivational level. That is, there was an animal in (*a*) withdrawal (i. e., motivated) which could by (*b*) moving its head to a certain position (an action) (*c*) obtain morphine or codeine to thereby reduce the withdrawal symptoms. These are the principles. Whether they can be applied to a different procedure and thus provide a further verification of their usefulness is the purpose of this second investigation.

To implement this investigation, an action, within the capabilities of the rat but not normally performed, was needed. Further, this action had to result in a morphine intake in some manner. An oral intake seemed to be the most feasible. Orally, morphine has a distinct bitter taste. Therefore, it was not unusual to find that rats, when offered a choice between a 0.5 mg./cc. aqueous morphine solution and water, drink large quantities of water but only 1 or 2 cc. of the morphine solution during a twenty-four-hour period. Thus, drinking the bitter morphine solution is within the capabilities of the rat, but it is also an action not normally made. Hence, it was decided that the action of drinking this bitter solution would be required of the animals. Then, if the animals are (*a*) in withdrawal (i. e., motivated) and they (*b*) drink the morphine solution (the action to be learned), then this would (*c*) reduce their withdrawal symptoms (i. e., reduce their motivational level). In view of this sequence of events, it would then be expected that the action of drinking the bitter solution would be learned by the animals.

The motivational state produced by the withdrawal syndrome may be considered a noxious stimulus. Learning an action which reduces a noxious stimulus is called "escape training."

EXPERIMENT I

To obtain motivated animals (i. e., animals in withdrawal), 12 female Sprague-Dawley rats, weighing between 150 and 190 Gm. were administered preliminary intraperitoneal injections of morphine hydrochloride daily for twenty-five days. The injections were begun at a dose level of 20 mg./Kg. and then increased 5 mg./Kg. daily thereafter.

To determine initial preference for the morphine solution, a preference test was made during the ninety-six-hour withdrawal period on days 26 to 29. The animals were individually caged in a temperature-controlled environment (72°). Free access was given to either tap water or tap water contain-

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ing 0.5 mg./cc. of morphine. Both solutions were presented in 100-cc. animal drinking tubes. A mean daily consumption of the morphine solution was found to be 0.9 cc. Even though the rats were motivated, they did not, during this pre-test period, learn the action of drinking the morphine solution. At least, the action was not performed to the extent that there resulted any appreciable reduction in the withdrawal symptoms.

At the end of the above pre-test period, the animals were randomly assigned to 2 groups of 6 rats each, an experimental and a control group. The experimental group was then given 10 escape training trials. One complete training trial consisted of twenty-four hours with no liquids (symbolized as *O*), twenty-four hours with morphine solution (*M*) and twenty-four hours with water only (*W*). The liquids were presented to each group in the same manner and under the same conditions as during the pre-test period. A series of training trials would be symbolized in this manner: *O, M, W, O, M, W, O, M, W*, etc. The control group was given 10 non-escape trials. A complete training trial for this group was identical to that of the experimental group except *W* was substituted for *M* in the cycle, to be symbolized in this manner *O, W, W, O, W, W, O, W, W*, etc.

During the first training trial, the mean morphine intake was 78 cc., which represented 149 mg./Kg. of morphine per rat. The morphine intake decreased during the second and third training trials, but at the fourth training trial, the mean consumption became stabilized at approximately 50 cc. mean morphine intake, or 96 mg./Kg. of morphine per rat. This amount, consumed every third day, was considered sufficient, as evidenced by the struggling of the animals when restrained, to maintain the withdrawal syndrome.

Four preference tests between water and morphine solution concluded this experiment. The tests, given to both the experimental and the control group, were conducted after the fifth and tenth training trials, after two weeks of complete withdrawal and finally, after seven weeks of complete withdrawal.

In explanation of the procedure selected for the experimental, it should be mentioned that withdrawal distress increases to a maximum between forty-eight and ninety-six hours after the last dose of morphine (4, 5). For this reason, the experimental group was presented with morphine *only*, every third day. Opportunity to drink the morphine solution and thus escape the withdrawal distress occurred every third day and each opportunity constituted one training trial. In order to insure that the rats would drink this normally avoided bitter fluid, water was removed for twenty-four hours from the caged animals, and morphine solution alone was presented for the next twenty-four hours. (The effect of thirst on learning this action is evaluated in a second experiment.)

In order to eliminate any group differences attributable to differences in the amount of morphine received by the two groups, the control animals were arbitrarily paired with the experimental animals. On the day following the drinking of the morphine solution by the experimental rats, the control rats were orally administered the same amount of morphine as the paired experimental animal drank. Thus, any differences in the results of the preference tests may not be attributed to a difference in morphine intake between the 2 groups.

EXPERIMENT II

In analyzing the results of Experiment I, there arose the question as to whether the results were effected through the animals associating the bitter taste of the morphine solution with the reduction of thirst motivation. If the results of the foregoing experiment were influenced by such an association, then similar learning should occur to an equally bitter quinine solution, or equally distasteful alum solution.

To investigate the possibility of this association, a quinine solution (*Q*) was equated with a morphine solution (*M*), by placing 5 naive female Sprague-Dawley rats, 150 to 200 Gm., in a cage with a choice of drinking a 0.5 mg./cc. morphine solution or a quinine solution. Starting with a weak solution of *Q*, the strength was increased and the tube positions changed daily until the rats drank more *M* than *Q*. This change in choice occurred abruptly between *Q* concentrations of 0.5 and 0.6 mg./cc.

To test the equation, 5 more naive female Sprague-Dawley rats, 150 to 200 Gm., were placed in a cage with a choice between solutions of 0.5 mg./cc. *M* and 0.5 mg./cc. *Q*. Only a very insignificant amount more of *Q* than *M* was consumed. The equation was accepted as sufficiently close for the experiment. In a similar manner, alum was equated at 40.0 mg./cc. solution.

Eighteen Sprague-Dawley rats of mixed sex, weighing 150 to 200 Gm. were then administered preliminary intraperitoneal injections of morphine hydrochloride on a schedule duplicating that of Experiment I. The 17 surviving rats were randomly assigned, 6 to a morphine group (*M*), 6 to a quinine group (*Q*) and 5 to an alum group (*A*). The morphine group was subjected to the training cycles of the morphine group of Experiment I. The quinine group was treated in a like manner except that the rats received a quinine solution to drink instead of a morphine solution, and similarly, the alum group received the alum solution to drink.

Again, to eliminate possible group differences on the preference tests, attributable to differences in the amount of morphine received by the groups, injections were administered to the *Q* group and the *A* group animals on the day following the presentation of their test solutions. As a matter of convenience, the rats in the *Q* and *A* groups were injected with the same amounts as the control group of Experiment I. These groups, therefore, received a very slight amount more morphine than the experimental group.

RESULTS

Figure 1 shows the change in preference for the bitter morphine solution by the two groups in Experiment I. Significance of the difference between the groups is given in Table I. Because of skewed distributions and because experimental and control rats were paired on the basis of amount of morphine received during the experiment, Wilcoxon's (6)

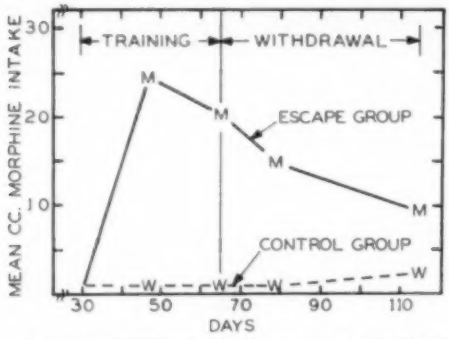

Fig. 1.—Experiment I. Mean cc. of morphine consumed on 4 preference tests by "escape trained" group (solid line), and by a "no escape" group (dash line).

paired replicate T test was used to obtain the indicated significances.

During the preference test following two weeks withdrawal, the two rats which drank the most M (more than 110 mg./Kg.) were the only animals which behaved differently just after the M tube was presented. They darted rapidly about the cage and then returned immediately to consume small amounts of the morphine solution.

Three rats died prior to the fourth preference test. The reduced number of the 2 groups made statistical significance impossible to obtain on this test, regardless of the scores of the remaining rats.

TABLE I.—GROUP DIFFERENCES ON PREFERENCE TESTS[a]

	p OF THESE PREFERENCES			
Test Number	Experiment I	Experiment II		
	$M = W$	$M = Q = A$	$M = Q$	$M = A$
1	0.05	0.05	0.05	0.05
2	0.05	0.005	0.01	0.01
3	0.05	NS	NS	NS

[a] All differences on preference test 4, as well as all differences between Q and A are nonsignificant.

Figure 2 gives the change in preference for the test solutions used in Experiment II. Significances of the differences are given in Table I. The overall probabilities were computed by the Kruskal-Wallis H test (7). Individual comparisons were computed with Wilcoxon's (6) unpaired replicate T test. One rat in the A group died prior to the fourth preference test.

DISCUSSION

As may be seen from Figs. 1 and 2, all differences between the means of the experimental and control groups favor the experimental groups, even after more than a month of complete withdrawal from morphine. These differences may *not* be attributed to any morphine-induced biochemical or physiological changes alone, since the control groups received as much or more morphine as the experimental groups.

The consistently low consumption of Q and A in all the preference tests of Experiment II refutes the possibility that association of taste and thirst may explain the results of Experiment I.

There is a significant difference ($p = 0.05$) between the morphine groups of Experiments I and II on the first preference test. Any of several differences between the 2 experiments may account for this difference in results (e. g., sex of animals, difference in stage of withdrawal at the start of the experiment, etc.).

The significant difference after two weeks withdrawal, as shown by the third preference test of Experiment I, is considered especially noteworthy. Wikler (5) reports that in humans, the withdrawal syndrome subsides gradually to a very low level after about one week of withdrawal. Stanton (8) gives graphs showing that rats, given four weeks of injections and then withdrawn, struggle at high levels when restrained. This struggling gradually subsides to preinjection levels after ten to twelve days of withdrawal. Thus, the withdrawal syndrome appears to diminish and asymptotically approach normality over a seven to twelve-day period. The significant difference between the groups after fourteen days withdrawal must, therefore, have been obtained after the withdrawal syndrome had reached an extremely low level. On this test, two rats exceeded 110 mg./Kg. during the twenty-four-hour period.

Even after thirty-five more days of withdrawal in Experiment I, 1 rat drank 42 mg./Kg. and another, 32 mg./Kg. These rats consumed an appreciable amount (their first injection was only 20 mg./Kg.) of the normally refused alkaloid when they had the option of drinking plain water and when their physical dependence upon morphine was certainly of an extremely low magnitude. These results and the results of the third preference test (after two weeks withdrawal) appear to be a relapse by these animals to the use of morphine.

The results of these experiments agree with the predictions of the escape training hypothesis. Since escape training is a form of conditioning, the habit of taking the drug would be expected to be strengthened each time the drug is taken to escape the with-

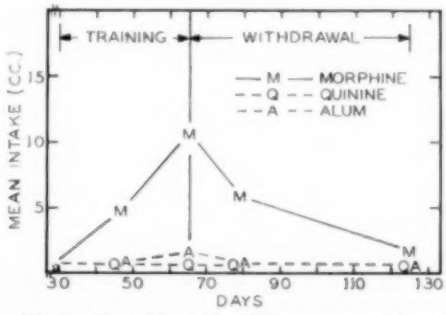

Fig. 2.—Experiment II. Mean cc. consumed on 4 preference tests by "escape trained" group (morphine solution) and by 2 "no escape" groups (quinine and alum solutions).

drawal syndrome. The noxious stimulus of withdrawal follows each use of the drug, and therefore, a cyclical behavior pattern is set up with each cycle of withdrawal and escape, strengthening the act of taking the drug.

What relation does escape training have to some of the other variables of addiction? Withdrawal has been identified as a significant factor by several investigators, e. g., Lindesmith (4) and Spragg (9). Physical dependence has been assumed to be the essence of addiction by some authors, e. g., Hesse (10), but has been rejected as a complete explanatory principle by others (4, 5, 9). Physical dependence and withdrawal are *necessary* but not *sufficient* for escape training to occur, and thus, are not sufficient for addiction to occur. This is well illustrated by hospitalized patients who may become physically dependent upon opiates, are withdrawn to suffer a classical withdrawal syndrome, and yet never relapse (4, 5). Since physicians routinely conceal from the patient the information that he is receiving opiates, the patient has no opportunity to learn that the symtoms of withdrawal are not part of his disease, but symptoms which can be banished by some action on his part, such as inducing the physician to give him another dose of the opiate. Once withdrawn and released from the hospital, these patients do not exhibit a compulsion, or "craving" in relation to the drug because they are not conditioned. It is not without significance that there is such a close parallel between the addictive liability of drugs and their ability to produce physical dependence. The greater the physical dependence, the greater the withdrawal symptoms and thus, the more highly motivated is the animal. And motivation is directly correlated with learning efficiency.

Personality factors, social factors, and availability lose none of their significance in addiction if the escape training hypothesis is accepted. Certain personality types would be more likely than others to try drugs, or to perceive drugs as a solution to their neurotic anxieties. There is even some possibility that there is a personality reorientation around the changed motivation supplied by addiction.

Physical dependence, tolerance, and withdrawal symptoms have been demonstrated many times in animals although relapse has not. Several difficulties are present in an attempt to condition animals. There is a relatively long time interval (in terms of conditioning) between performance of the action of escape and the physiological changes constituting an escape. Language is an important factor in bridging this gap for man, as Lindesmith (4) has pointed out. This is a reflection of man's superior learning ability and his superior instrument of learning which is language. Animals, of course, are severely handicapped in this area.

The orientation of previous experiments with animals has been such as to increase an already difficult conditioning situation. For example, Spragg's (9) chimpanzees were required to perform a variety of actions for an injection of morphine.

Confirmation of the predictions of the escape training hypothesis is important in several ways. A rationale is provided for many otherwise discrete phenomena of addiction. The working hypothesis that physical dependence and withdrawal are somehow related to addiction is substantiated. This implies laboratory estimation of the addictive liability of new drugs by measuring their tendency to produce withdrawal symptoms which can be relieved by more of the drug may be possible. A "hair of the dog that bit you," i. e., an alcoholic drink, is a well known method of alleviating a "hangover." It has also been shown to be an early feature of compulsive drinking (11).

The efficiency of escape learning has been demonstrated. Only three weeks of injections and ten days of escape training produced learning in rats which was still apparent after thirty-five days withdrawal. Further work is in progress on some of the implications of this research.

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The Polymerization of Pharmacophoric Moieties and Its Effect Upon Biologic Activity I.*

Polymeric Quaternary Ammonium Salts

By F. W. SCHUELER† and H. H. KEASLING

A group of polymeric quaternary ammonium salts have been synthesized and studied pharmacologically. The polymers exhibited a high degree of neuromuscular blocking potency. The results have been discussed both in regard to their theoretic and practical importance. Such polymers exemplify the idea that multiplication of pharmacophoric moieties through polymerization may yield drug agents having very long durations of action.

THIS REPORT has the specific objective of detailing the pharmacologic effects of certain polymeric quaternary ammonium salts. It takes as a general objective the initiation of some research concerning the alterations in pharmacologic activity that occur when given pharmacophoric moieties are multiplied in linear, planar, and three-dimensional molecular matrices. It is felt that these studies yield information pertinent to the effect that spatial distribution of chemical groups has upon drug action.

EXPERIMENTAL

Chemical Syntheses.—The products synthesized together with their associated physical properties and analyses are summarized in Table I. The methods used in the synthesis of specific materials are illustrated below under the headings "simple products" and "polymers."

Simple Products.—The method used in the synthesis of all bisquaternary ammonium salts (I, III, V, in Table I) is illustrated by the preparation of N,N bis-*p*-(trimethylammonium) xylylene dibromide.

A hot alcohol solution containing a 1 *M* proportion of *p*-xylylene dibromide was poured into a round bottom flask containing an excess of trimethylamine (25% in alcohol) solution. The mixture was refluxed two hours on a water bath, cooled, and poured with stirring into a fourfold volume of acetone. The crystals deposited were recrystallized repeatedly by resolution in alcohol followed by precipitation in acetone until a constant melting point was obtained. Yields in the case of all of these salts were above 90%.

Polymers.—The polymeric quaternary ammonium products containing aromatic rings (II, IV, VI, of Table I) were obtained by mixing in hot alcohol solution equal molar proportions of the appropriate dibromides and bis-tertiary amines, [e. g., *p*-xylylene dibromide and bis-*p*-(dimethylamino) xylylene]. The mixtures were refluxed for not less than four hours and poured with rapid stirring into excess (4× volume) of acetone. The polymers deposited rapidly as white, nonhygroscopic solids. All polymers were redissolved in alcohol followed by precipitation in acetone. Estimates of the average degree of polymerization were obtained through analyses of ionizable and nonionizable bromide as employed by Marvel, *et al.* (1).

The polymethylene polymer (VIII in Table I) was obtained following the precise procedure indicated by Marvel, *et al.* (1), for this compound. Decamethylene bis(trimethylammonium) dibromide (VII in Table I) was obtained from commercial sources.

Pharmacologic Procedures

Toxicity Determinations in Mice.—Toxicity experiments on white mice were carried out on all new products and, in certain cases, the joint toxicity of simple products with various polymers was also determined. All products were injected intraperitoneally after solution in 0.9% sodium chloride solution.

Experiments on Dogs.—Dogs under pentobarbital sodium (36 mg./Kg. administered i. v.) were prepared for the recording of respiration, left carotid arterial blood pressure, and contractions of the gastrocnemius muscle during electric stimulation of the sciatic nerve. Parameters for nerve stimulation were fixed through the use of a Grass Model S4A stimulator. Voltage, which was always supramaximal, ranged from 5–50 volts with square wave monophasic pulse bursts having a frequency of 250/sec. induced for 0.1 sec. at intervals of 10 seconds. The vagus nerve was isolated on the right side, sectioned, and an electrode arranged for peripheral stimulation with an inductorium.

Following the establishment of control responses, [(i. e., occlusion of the uncannulated carotid artery (10 seconds), vagal stimulation (5 seconds) and epinephrine, histamine, and acetylcholine, all administered i. v. at the dose level 1 μg./Kg.)] a dose of the drug under test was administered i. v. Following a suitable period, during which the effects of the drug alone were observed, the control procedures were repeated. Increasing doses of the drug under test followed by the test procedures were continued in an alternating manner. In cases where a dose of the test drug induced respiratory

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paralysis, oxygen was administered endotracheally via a fine plastic catheter until respiration resumed or until the experiment was terminated.

Experiments on Chickens.—Chickens were prepared for the recording of contractions of the gastrocnemius muscle under electric stimulation of the sciatic nerve according to the directions of Thesleff and Unna (2). Drugs were administered i. v. in saline following the establishment of a constant level of contraction height to supramaximal stimulation.

ity tests (Tables II and III) were: (*a*) polymerization of the "short" (8Å.) monomers greatly increases their toxicity, (see A and B in Table V); (*b*) polymerization of the "long" (15.8–16.0Å.) monomers is followed by a decrease in toxicity, (see C and D in Table V); and (*c*) previous administration of a "low" dose of a given agent may either supplement or decrease the toxic effect of a subsequently administered agent (Table III). No obvious generalization was made apparent, however, through these tests.

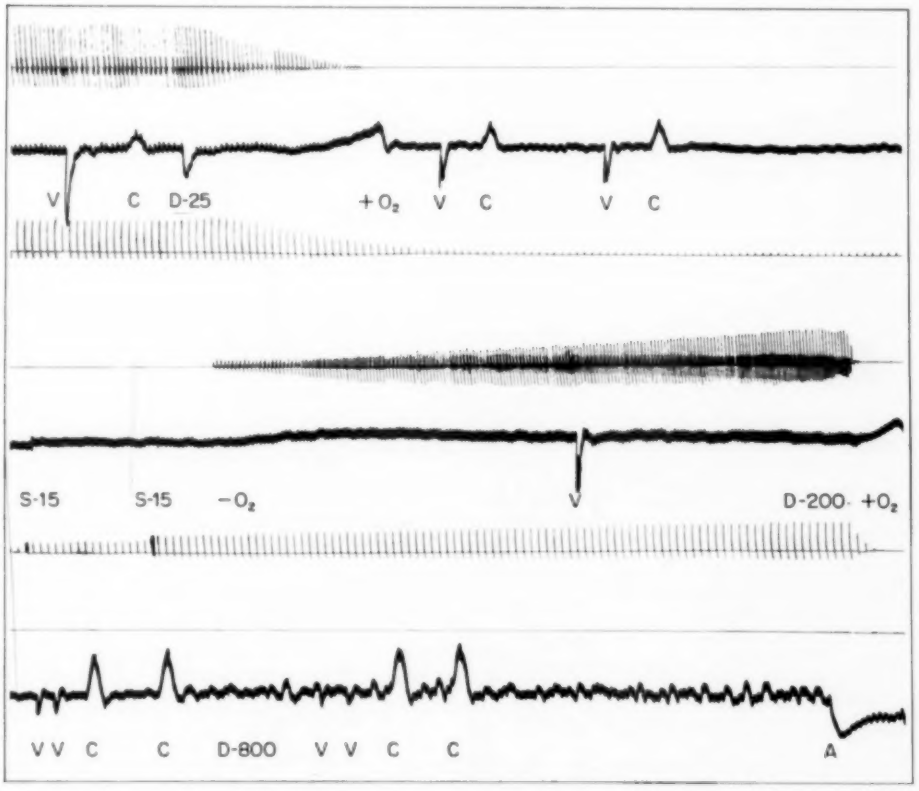


Fig. 1.— *V* = vagal stimulus for 5 seconds, *C* = right carotid occluded 10 seconds, *D-* = drug no. IV and dose in μg./Kg., $+O_2$ = endotracheal oxygen, *A* = acetylcholine 10 μg./Kg., *S*-15 = stop rotation of kymograph for 15 seconds. The three kymograph tracings at the top of the figure are: respiration, blood pressure, and gastrocnemius contractions, in the order named.

RESULTS AND DISCUSSION

Referring to Table I, it is convenient to view the various agents according to the following monomer-polymer relationships: (A) II as a polymer of I, (B) IV as a polymer of III, (C) VI as a polymer of V, and (D) VIII as a polymer of VII. Also of interest in this discussion are the intramolecular distances between adjacent nitrogen atoms as summarized in Table V.

The main conclusions to be drawn from the toxic-

Data from the experiments utilizing dogs are summarized in Table IV. The compounds (I–VI) produced respiratory failure accompanied by marked depression of neuromuscular transmission and blockade of the vagal depressor response.

In no case were modifications in the test responses to acetylcholine or histamine observed. Agents I and III, at doses which produced minimal neuromuscular depression, were effective in producing ganglionic blockade as indicated by their ability to depress the response to occlusion of the right carotid artery.

TABLE I.—STRUCTURES, PHYSICAL PROPERTIES, AND ANALYSES

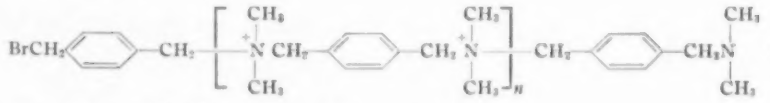
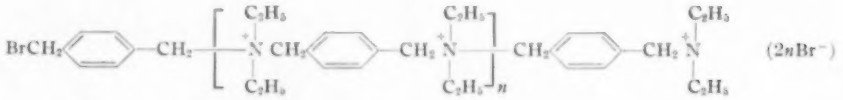
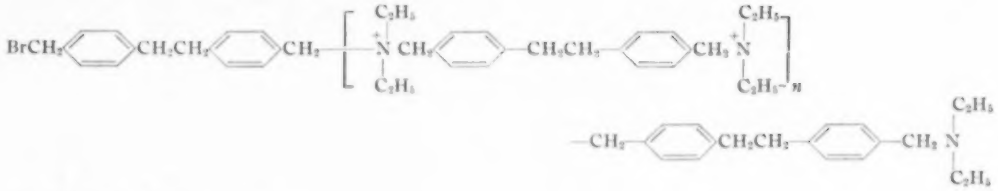
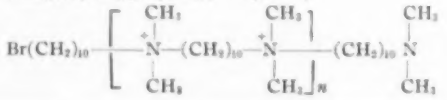
Product No.	Structure	M. P. (Uncorr.)	Analysis and Average (n) for Polymer
I		>295	Calcd. % Br^- 41.6 Fnd. % Br^- 41.4
II		270–285 (decompn.)	$n = 30$
III		>295	Calcd. % Br^- 34.2 Fnd. % Br^- 34.0
IV		265–268 (decompn.)	$n = 22$
V		>295 (decompn.)	Calcd. % Br^- 28.9 Fnd. % Br^- 28.4
VI		>295 (decompn.)	$n = 8$
VII		...	...
VIII		204–208	$n = 37$

TABLE II.—TOXICITY OF INDIVIDUAL AGENTS

Product No.	Estimated LD_{50} (mg./Kg.)	No. Mice Used
I	50.0	65
II	1.5	58
III	15.0	52
IV	0.75	26
V	1.5	124
VI	10.0	103
VII	0.9	25
VIII	1.8	25

TABLE III.—JOINT TOXICITY DATA

Product No.	Dose, mg./Kg.	Killed, %	Effect of Combination on Toxicity
I	20.0	0.0	Increased
II	1.5	45.0	
I/II[a]	20/1.5	69.0	
II	0.5	0.0	Increased
I	60.0	92.0	
II/I	0.5/60	100.0	
III	10.0	.0	Decreased
IV	0.625	38.0	
III/IV	10/0.625	23.0	
IV	0.1	15.0	Decreased
III	20.0	92.0	
IV/III	0.1/20.0	38.0	
V	0.25	0.0	Increased
VI	12.5	6.0	
V/VI	0.25/12.5	100.0	
VI	5.0	0.0	...
V	2.0	100.0	
VI/V	5.0/2.0	100.0	
I	20.0	0.0	Decreased
IV	0.625	38.0	
I/IV	20/0.625	15.0	

[a] The numerals (e. g., I/II) should be read as follows: a low dose of I was followed by a high dose of II. In such cases it is seen that the previously administered drug may or may not increase the effect of the subsequently administered agent.

In addition they caused blockade of the vagal depressor response with an *increase* in the pressor response to carotid occlusion! Since the effects of acetylcholine injection were unaltered by II and IV, they do not act as postganglionic parasympathetic blocking agents. Though no complete proof of the conjecture can be offered at this time, it is suggested that these agents block in some way parasympathetic ganglia in the heart only. The nonane derivative described by Kensler, *et al.* (3), may represent a precedent for this type of action. Compounds VII and VIII were not compared in the dog due to the limited supply of the polymer VIII available for this study.

All polymers (II, IV, VI, VIII) were studied for their neuromuscular blocking actions in the sciatic nerve gastrocnemius muscle preparation of the chicken. All of the polymers produced a flaccid type of paralysis antagonizable by neostigmine or Tensilon® (Hoffmann-La Roche Inc.). The polymers were exceptionally interesting in this type of test since each produced neuromuscular blockade of very great duration (Table VI). Even in the case of the polymer VIII, which was less toxic in mice than its monomeric analog VII, the duration of effect of the polymer VIII was many fold that of the monomer VII (decamethonium). We are at a loss to explain the fact that even the polymer of decamethonium, compound number VIII, yields a flaccid paralysis. These increases in duration of action may be a result of the increased time required for excretion of large polymeric substances or because the larger molecules are bound more securely to the receptors. Experiments designed to test these alternative hypotheses are in progress. Whatever the mechanism of the increase in duration of effect it is of obvious practical interest, especially in pharmaceutical chemistry, for it suggests that polymerization of moieties may be a useful way to increase the duration of action of drugs.

TABLE IV.—DATA FROM EXPERIMENTS ON DOGS[a]

Product No.	Dose mg./Kg.	% Effect of Product Upon Resp.	B. P.	M. C.	% Alteration of Test Response After Product C. O.	V.	E.	Remarks
I	2.0	↓ 20	↓ 25	...	↓ 50	↓ 90	↑ 10	
	8.0	↓ 60	↓ 45	↓ 10	↓ 100	↓ 100	↑ 20	
	16.0	↓ 95	↓ 60	↓ 85	↓ 100	↓ 100	↑ 10	O_2 administered during resp. arrest
II	0.030	↓ 60	↓ 20	↓ 80	?	↓ 100	↑ 10	
	0.100	↓ 100	↓ 25	↓ 100	↑ 10	↓ 100	↑ 10	O_2 administered during resp. arrest
III	2.0	↓ 25	↓ 10	↓ 10	↓ 20	↓ 80	↑ 5	
	4.0	↓ 90	↓ 20	↓ 90	↓ 75	↓ 100	↑ 10	O_2 administered during resp. arrest
IV	0.025	↓ 100	↓ 5	↓ 100	↑ 10	↓ 60	↑ 5	O_2 administered during resp. arrest
	0.100	↓ 100	↓ 10	↓ 100	↑ 25	↓ 80	↑ 10	O_2 administered during resp. arrest
V	3.0	↓ 100	..	↓ 100	↓ 15	↓ 90	..	O_2 administered during resp. arrest
VI	0.400	↓ 90	↓ 10	↓ 85	↓ 20	↓ 95	↑ 15	O_2 administered during resp. arrest

[a] ↑ denotes increase, ↓ decrease, Resp. denotes amplitude of respiration, B. P. denotes carotid arterial blood pressure, M. C. denotes amplitude of muscle contraction, C. O. denotes carotid occlusion, V denotes vagal stimulation, and E denotes response to 1 μg./Kg. epinephrine.

Most striking during the experiments on dogs were the results obtained with the polymers II and IV as illustrated in Fig. 1. Both of these materials effected neuromuscular blockade at exceedingly low doses (0.025–0.030 mg./Kg.). It was also noted that the agents II and IV were more effective in blocking neuromuscular transmission involved in respiration than on the sciatic gastrocnemius junction. This is, of course, in striking contrast to the action of decamethonium and *d*-tubocurarine.

Referring to Table V, it will be noted that in all cases the polymerization of the agents having the internitrogen distance 8Å., (i. e., I to II in A, III to IV in B) yields an increase in neuromuscular blocking potency and duration. On the other hand, polymerization in the case of agents having the internitrogen distance 15.8Å. or 16.0Å., (i. e., V to VI in C, VII to VIII in D) yields no increase in

neuromuscular blocking potency (indeed a decrease is observed) though a striking increase in the duration of action is still observed. If one presumes that optimum neuromuscular blocking action is obtained in materials having an internitrogen distance of 16Å., it would appear that the increase in neuromuscular blocking potency induced by polymerization in situations A and B, is due to the fact that such a distance is possible in those polymers and not in their monomers.

TABLE V.—INTRAMOLECULAR DISTANCES BETWEEN NITROGEN ATOMS

Monomer-Polymer Relationship	Compound No. from Table I	Maximum Distance (Å.) Between Adjacent Nitrogen Atoms[a]
A	I	$8.0 \times m$[b]
	II	$8.0 \times m$
B	III	$8.0 \times m$
	IV	$8.0 \times m$
C	V	$16.0 \times m$
	VI	$16.0 \times m$
D	VII	$15.8 \times m$
	VIII	$15.8 \times m$

[a] Measurements made using Fischer-Hershfelder-Taylor atomic models.
[b] m represents an integer. It is obvious that m may exceed the average n as indicated in Table I.

fractions lying in different length ranges, can determine the length-*versus*-potency relationships actually involved.

The high potency of the various polymers in the present study suggests the *possibility* that a given number of moieties within a single molecule may be as effective in their combination with the receptor sites of cells as the same number of moieties contained in a population of smaller individual molecules. If this contention be borne out by future investigation such polymers may have value as tools in the mapping of the lattice work of the receptor sites as they occur on or within cells.

SUMMARY

A series of polymeric quaternary ammonium salts together with their monomeric analogs have been synthesized and studied for their relative pharmacologic effects in several preparations. The results suggest that the polymerization of pharmacophoric moieties may be a useful practical procedure for increasing the duration of action of drugs. The results also suggest that polymerization of pharmacophoric moieties may yield products of considerable interest in the analysis of drug-receptor relationships.

TABLE VI.—DATA FROM CHICKEN PREPARATION

	Dose (mg./Kg.) to 10% or Less of Control Contraction Height	Type of Paralysis	Antagonism by Neostigmine and Tensilon	Duration (min.) or Time Till Return of at Least 90% of Control Contraction Height
I	~50.0	Flaccid	+	10–15
II	0.5	Flaccid	+	>180
III	~50.0	Flaccid	+	10–15
IV	0.5	Flaccid	+	>180
V	1.0	Flaccid	+	24
VI	1.0	Flaccid	+	>180
VII	0.050	Spastic	−	8
VIII	0.200	Flaccid	+	>90

It must be kept in mind that while the average length (Table I) of the various polymers is quite large, the present study allows for no estimation of the proportion of dimers, trimers, or other small polymers present in the polymeric agents which are necessarily mixtures. It is, therefore, conceivable that the main activity of the polymers is due to the shorter members of the molecular population. Only separation of the shorter molecular species from the larger polymer molecules by dialysis or other methods, together with pharmacologic tests of the

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The Influence of Various Agents Upon the Intensity and Duration of Morphine Analgesia in Rats*

By BERNARD LEVY and L. D. EDWARDS†

Nine compounds were tested for their ability to increase peak intensity of effect or prolong the analgetic activity of morphine sulfate in rats. A modification of the Wolff, Hardy, and Goodell heat stimulus method was used. Urethan produced increases only to a slight extent. Atropine sulfate, polyvinylpyrrolidone (PVP), and dextran increased peak intensity of effect and prolonged the analgetic action of morphine sulfate. PVP was the most effective agent used in this study. Of five diphenyl derivatives tested, Transentine® was very effective and 1-diphenyl-4-dimethyl-amino-propyl pentanoate markedly so. Benadryl® and Histadyl® were also effective, but Chlor-Trimeton® was one of the most effective diphenyl derivatives studied. Administration of morphine sulfate in solutions of PVP following pretreatment of the rats with Transentine, Histadyl, or Chlor-Trimeton produced a greater increase in the peak intensity of effect and also a greater prolongation effect than any of these compounds when given alone.

In cases of intractable pain the use of the analgetic drugs is widespread. Of these drugs morphine is the most commonly employed. The use of morphine as an analgetic agent is not above criticism. For example, it has a transient duration of action since it lasts only two to three hours after administration by the parenteral route thus necessitating frequent injections. Morphine exerts a depressant effect on the centers of respiration resulting in a decreased minute volume of gaseous exchange. The overall effect of morphine on the gastrointestinal tract is constipative. Also with morphine therapy is the ever present danger of addiction.

The vast majority of research that has been conducted with morphine has had as its ultimate goal the development of an effective morphine substitute. Comparatively little work has been done to find a method of increasing the peak intensity of effect of morphine itself. It is the objective of this study to find a drug or combination of drugs that will effectively increase the peak intensity of effect or prolong the analgetic activity of morphine. Such a drug or combination of drugs would thus either allow a smaller dosage of morphine or less frequent injections.

EXPERIMENTAL

The method for measuring the pain threshold, originally described by Wolff, Hardy, and Goodell (1), was utilized in the construction of the apparatus used in this study. The apparatus consisted of a photospot lamp (G. E. No. RSP2 115-120V), variac, timer, signal key, and a voltmeter connected in series. Pressing the signal key turned on the lamp and started the timer simultaneously. An ordinary heat lamp reflector was used to shield and to focus the light producing a spot of light of intense heat about 1.3 inches in diameter. The voltmeter was included in the circuit to determine whether or not fluctuations in the line voltage occurred. The beam of light was focused on the tip of the rat's tail, previously blackened with India ink.

The rats were immobilized by the use of nylon mesh holders. The nylon mesh holders were sewed in the shape of small bags approximately 3 inches wide and 8 inches long. By placing the animal in this type of holder and then tacking the holder to a board the animal was firmly but comfortably restrained. The animal remained in the holder for the length of the experiment thus eliminating excessive handling by the operator.

Preliminary experiments were carried out to determine the constancy of the results obtained with this apparatus on normal, untreated rats. These experiments also served to show what voltage would be needed to produce an average reaction time of between three and five seconds. Sprague-Dawley rats of both sexes weighing between 85 and 200 Gm. were used in all studies. The procedure used in determining normal reaction times was as follows: the animals were placed in the holders with the tail protruding. The tails of the animals were washed with tepid water and dried and the last $1^1/_2$ inches painted with India ink. After a priming stimulation, four reaction times were determined, each after an interval of ten minutes. The end point was taken as the first perceptible movement of the rat's tail. A group of 21 rats was used in this control experiment.

In determining the ability of the various agents studied to prolong or increase the peak intensity of effect of morphine analgesia, the following routine was observed. Three rats would receive morphine in aqueous solution and the other three would receive morphine plus the agent under investigation. Two days later this routine was reversed on the same group of rats. Thus each rat served as its own control. All injections were given subcutaneously.

* Received March 29, 1956, from the Purdue University School of Pharmacy and the Medical College of Georgia, Augusta.

† Deceased.

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Light intensity was controlled by means of the variac. Voltage was adjusted so that the majority of animals respond in three to five seconds; those not giving three consecutive responses within this interval were discarded. Cutoff time was ten seconds or approximately two and one-half times the average reaction time. A reading of ten seconds indicated complete analgesia. The results thus obtained were tabulated by reporting the mean reaction times for each group of rats at the specified time intervals after the administration of morphine alone and morphine plus an agent under investigation.

TABLE I.—SUMMARY OF THE EFFECTS OF URETHAN UPON MORPHINE ANALGESIA (RAT)

Drug mg./Kg.	No. Rats	Mean Reaction Time, in Seconds, Taken at ½-Hr. Intervals				
		½	1	1½	2	2½
Morphine 10 alone	6	5.4	5.3	4.7	3.8	3.6
Morphine 10 + urethan 250	6	8.0	7.2	6.4	5.8	5.8
Morphine 10 alone	6	7.6	7.2	6.0	4.5	4.0
Morphine 10 + urethan 350	6	8.6	8.7	7.9	7.4	6.7
Urethan 350 alone	6	3.5	3.6	3.6		

The Effect of Urethan Upon Morphine Analgesia.—Morphine is thought to be oxidized to an inactive form in the liver under the influence of the enzyme tyrosinase (2). Urethan has been reported to be capable of inhibiting this enzyme system (3–5). Morphine is also excreted to a certain extent in the form of the glucuronide. Conjugation is believed to take place in the liver. Therefore, agents capable of inhibiting glucuronic acid formation might possibly increase the peak intensity of effect of morphine analgesia. Urethan is said to be capable of inhibiting the formation of glucuronic acid and represents an additional reason for its use in this study (6). The results of the administration of urethan with morphine sulfate are shown in Table I.

The Effect of Polyvinylpyrrolidone (PVP) and Dextran Upon the Depth and Duration of Morphine Analgesia.—PVP has been reported to be capable of prolonging the vasodepressor activity of hexamethonium bromide by slow liberation of this agent after subcutaneous administration (7). It is for this reason that PVP and dextran, another blood expander, were employed in this study. The results of the administration of PVP and dextran are summarized in Tables II and III.

The Effect of Some Diphenyl Derivatives Upon the Depth and Duration of Morphine Analgesia.—The introduction of the experimental chemicals

TABLE II.—SUMMARY OF THE EFFECTS OF DEXTRAN UPON MORPHINE ANALGESIA (RAT)

Drug, mg./Kg.	No. Rats	Mean Reaction Time, in Seconds, at ½-Hr. Intervals								
		½	1	1½	2	2½	3	3½	4	4½
Morphine 12	6	7.0	8.9	7.1	6.1	3.9	3.1			
Morphine 12 + dextran 25%	6	7.7	10	10	10	8.5	6.8			
Morphine 15	6	9.3	9.4	9.0	7.8	6.3	5.2	4.5	4.8	4.1
Morphine 15 + dextran 25%	6	9.6	10	10	9.4	8.9	8.8	8.7	8.5	3.8
Morphine 12	6	9.0	9.3	9.3	6.2	6.2	4.2	5.2	4.4	
Morphine 12 + dextran 45%	6	6.4	9.9	10	10	9.6	9.2	7.7	4.8	
Morphine 15	6	10	10	10	9.6	5.8	4.7	3.9		
Morphine 15 + dextran 45%	6	9.6	10	10	10	8.8	6.5	4.2		
Morphine 15	6	10	10	9.1	8.0	5.4	3.3			
Morphine 15 + dextran 10%	6	9.4	9.2	9.3	8.9	6.7	3.8			
Morphine 12	6	7.5	8.1	7.0	5.4	4.8	4.1			
Morphine 12 + dextran 10%	6	8.6	8.5	9.3	7.6	5.8	4.5			

TABLE III.—SUMMARY OF THE EFFECTS OF PVP UPON MORPHINE ANALGESIA (RAT)

Drug, mg./Kg.	No. Rats	Mean Reaction Time, in Seconds, at ½-Hr. Intervals								
		½	1	1½	2	2½	3	3½	4	4½
Morphine 15	6	8.6	8.5	6.8	6.3	5.8	5.0	4.2	3.5	4.4
Morphine 15 + PVP 20%	6	9.6	10	10	10	10	10	9.8	7.0	5.4
Morphine 12	6	7.1	6.7	5.3	4.7					
Morphine 12 + PVP 20%	6	7.5	10	9.6	8.0	6.9	4.8			
Morphine 5	6	5.2	5.7	4.7						
Morphine 5 + PVP 20%	6	5.6	7.7	6.1						
Morphine 12	6	7.8	7.8	6.2	4.8	4.1	3.2			
Morphine 12 + PVP 40%	6	8.7	10	10	10	10	8.9	7.7	4.6	
Morphine 15	6	8.8	8.6	8.8	8.5	6.2	4.9			
Morphine 15 + PVP 40%	6	9.5	10	10	10	10	9.8	9.2	9.0	7.1
Morphine 12	6	8.5	8.7	7.8	4.3					
Morphine 12 + PVP 25%	6	9.2	10	9.4	9.3	7.9	7.0	6.3	5.4	
Morphine 15	6	8.5	9.6	10	8.7	6.6	4.4			
Morphine 15 + PVP 25%	6	8.5	10	10	9.9	9.9	7.3	4.6		
Morphine 10	6	8.3	6.9	5.2	4.2					
Morphine 10 + PVP 25%	6	8.3	10	10	9.5	7.0	5.3			
PVP 40%	6	3.7	3.6	3.4						

SKF® 525-A and P®-19, two diphenyl derivatives, as general potentiators suggested the use of other similar compounds as possible potentiators of morphine sulfate. Trasentine, 1-diphenyl-4-dimethyl-amino-propyl-pentanoate, Benadryl, Histadyl, Chlor-Trimeton, and atropine were studied because of their similarity in structure to SKF 525-A and P-19. The results of this study are summarized in Tables IV and V.

Effect of Combinations of Drugs.—The most effective agents thus far determined were administered together in an attempt to produce even greater effects. Histadyl, Trasentine, and Chlor-Trimeton were administered together with PVP. The results are shown in Table VI.

TABLE IV.—SUMMARY OF THE EFFECT OF SOME DIPHENYL DERIVATIVES UPON MORPHINE ANALGESIA (RAT)

Drug, mg./Kg.	No. Rats	Mean Reaction Time, in Seconds at ½-Hr. Intervals						
		½	1	1½	2	2½	3	3½
Morphine 10 alone	6	7.5	7.6	8.4				
Morphine 10 + atropine 1	6	7.9	8.1	7.1				
Morphine 15 alone	6	8.4	8.6	7.6	6.9			
Morphine 15 + atropine 2	6	9.0	8.8	8.1	6.9			
Morphine 12 alone	6	5.9	8.7	6.1	4.1			
Morphine 12 + atropine 2	6	9.2	10	9.4	9.4	8.7	5.8	
Morphine 12 alone	6	6.5	6.4	6.3	5.0	4.3		
Morphine 12 + atropine 3	6	8.8	8.9	9.0	7.7	5.1		
Morphine 10 alone	6	7.6	7.7	7.7	5.6			
Morphine 10 + Trasentine 100	6	8.5	9.7	9.1	8.7	7.3	5.4	
Morphine 8 alone	6	5.9	6.5	6.4	4.5	3.6		
Morphine 8 + Trasentine 100	6	8.9	9.9	9.2	7.8	5.1		
Morphine 12 alone	6	8.2	9.3	8.6	7.9	6.0	4.0	
Morphine 12 + Trasentine 100	6	9.9	10	8.1	8.3	7.8	7.2	4.3

TABLE V.—SUMMARY OF THE EFFECT OF SOME DIPHENYL DERIVATIVES UPON MORPHINE ANALGESIA (RAT)

Drug, mg./Kg.	No. Rats	Mean Reaction Time, in Seconds, at ½-Hr. Intervals						
		½	1	1½	2	2½	3	3½
Morphine 10 alone	4	6.9	8.5	5.3	4.5			
Morphine 10 + Comer's Compound[a] 100	4	6.8	9.3	9.0	10	9.5	8.7	
Morphine 10 alone	4	7.3	9.6	8.4	6.6			
Morphine 10 + Comer's Compound 50	4	9.0	10	10	10	9.1	5.3	
Morphine 10 alone	6	9.4	10	7.5	6.8	5.2		
Morphine 10 + Benadryl 100	6	9.2	10	10	9.7	9.4	7.4	4.6
Morphine 10 alone	6	8.0	9.4	10	10	9.7	6.7	6.0
Morphine 10 + Histadyl 25	6	9.2	9.7	10	9.8	9.3	9.0	6.0
Morphine 10 alone	8	8.8	10	9.3	7.0	5.5	4.1	3.5
Morphine 10 + Histadyl 50	8	9.0	10	10	10	8.6	7.5	5.2
Morphine 10 alone	6	9.6	9.9	9.2	6.8	5.3	3.8	
Morphine 10 + Chlor-Trimeton® 15	6	9.3	9.7	10	9.8	9.4	6.5	
Morphine 10 alone	6	8.8	9.3	8.6	7.7	6.6	6.2	4.8
Morphine 10 + Chlor-Trimeton® 25	6	9.5	9.8	10	9.6	8.5	7.1	5.4

[a] 1-Diphenyl-4-dimethyl-amino-propyl pentanoate.

TABLE VI.—SUMMARY OF THE EFFECTS OF COMBINATIONS OF PVP WITH TRASENTINE, HISTADYL, AND CHLOR-TRIMETON UPON MORPHINE ANALGESIA (RAT)

Drug, mg./Kg.	No. Rats	Mean Reaction Time, in Seconds, at ½-Hr. Intervals								
		½	1	1½	2	2½	3	3½	4	4½
Morphine 12 alone	6	7.4	10	10	9.7	9.0	5.0	3.9		
PVP 25% + morphine 12 + Histadyl 50	6	7.9	10	10	10	10	8.9	7.3	6.2	
Morphine 12 alone	6	7.5	10	9.4	9.1	7.9	7.7	6.2	5.0	
Morphine 12 + PVP 25% + Trasentine 100	6	6.4	8.8	10	10	10	9.5	8.9	8.7	6.6
Morphine 10 alone	6	9.1	10	9.2	7.6	5.9	4.6			
Morphine 10 + PVP 40% + Trasentine 100	6	8.2	9.0	10	10	8.5	6.7			
Morphine 15 alone	5	9.0	10	8.8	8.2	6.0	4.0			
Morphine 15 + PVP 25% + Chlor-Trimeton® 15	5	7.9	10	10	10	10	8.3	6.9	5.5	
Morphine 12 alone	6	6.3	8.8	9.1	6.4	4.4	3.6			
Morphine 12 + PVP 25% + Chlor-Trimeton® 25	6	7.3	8.3	10	9.8	8.7	7.5	6.9	4.7	

SUMMARY

Nine compounds were tested for their ability to increase the peak intensity of effect or prolong the analgetic activity of morphine sulfate. A modified Wolff-Hardy-Goodell apparatus for the measurement of reaction time in rats was used to determine the intensity and duration of morphine analgesia. The authors wish to acknowledge the fact that the method employed is not the most sensitive method for measuring analgetic activity. This method is not capable of detecting mild analgetic activity, such as that exhibited by aspirin. However, it is capable of detecting gross differences in the analgetic activity of the narcotic analgetics. The results of this study are summarized below.

1. Urethan in a dosage of 250 mg./Kg. produced a slight increase in the peak intensity of effect of morphine sulfate. In a dosage of 350 mg./Kg. urethan produced both an increase in the peak intensity of effect and prolongation of analgesia. Urethan in a dosage of 350 mg./Kg., given alone, exerted no analgetic action.

2. Atropine sulfate in a dosage of 2 mg./Kg. produced both intensity of effect and prolongation of the analgetic activity of 12 mg./Kg. of morphine sulfate. Similar results were obtained with a dosage of 3 mg./Kg. of atropine sulfate.

3. PVP produced both an increase in peak intensity of effect and prolongation of the analgetic activity of morphine sulfate in all doses and concentrations employed. The degree of efficiency of this agent was greater than any other single compound studied. The peak of morphine analgesia was greatly prolonged. PVP is apparently nontoxic and would therefore represent a safe and effective method of augmenting morphine analgesia.

4. The administration of morphine sulfate in dextran produced an increase in peak intensity of effect of morphine analgesia in all concentrations used. The administration of morphine sulfate in 25% and 45% dextran solutions significantly prolonged the peak of morphine analgesia.

5. In a dosage of 100 mg./Kg. Trasentine produced an increase in the peak intensity of effect of morphine sulfate. The degree of increase in peak intensity of effect was greater with smaller doses of morphine sulfate. In a dosage of 100 mg./Kg. given alone, Trasentine exerted no analgetic activity of its own.

6. Comer's Compound, 1-diphenyl-4-dimethyl-amino-propyl pentanoate, in doses of 50 and 100 mg./Kg. markedly increased the peak intensity of effect of morphine sulfate. This compound is quite similar in structure to certain synthetic narcotic analgetics and further investigation of this compound is indicated.

7. Benadryl in a dosage of 100 mg./Kg. produced a definite increase in peak intensity of effect of the analgetic action of morphine sulfate. In a dosage of 150 mg./Kg. Benadryl gave rise to toxic symptoms.

8. Histadyl in a dosage of 50 mg./Kg. increased the peak intensity of effect of the analgetic activity of morphine sulfate.

9. Chlor-Trimeton in a dosage of 15 mg./Kg. significantly increased the peak intensity of effect of morphine sulfate in a dosage of 10 mg./Kg. In a dosage of 25 mg./Kg. Chlor-Trimeton produced an even greater increase in peak intensity of effect. Dosage of 40 and 50 mg./Kg. given thirty minutes prior to 8 and 10 mg./Kg. morphine sulfate, respectively, gave rise to toxic symptoms. Chlor-Trimeton given alone to a group of six rats in a dosage of 50 mg./Kg. exerted no analgetic activity of its own.

10. The administration of morphine sulfate in solutions of 25% and 40% PVP following pretreatment of the rats with Histadyl, Trasentine, or Chlor-Trimeton greatly increased the peak intensity of effect and prolonged the action of morphine sulfate. The significant point in this experiment was the prolongation effect. The administration of morphine sulfate in PVP coupled with pretreatment of the animals with these diphenyl derivatives appeared to produce greater prolongation than did any of these compounds when administered alone.

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A Pharmacological Study of Some Acetylenic Carbinols*

By WILLIAM J. KINNARD, Jr.,† EDWARD C. REIF, and JOSEPH P. BUCKLEY

A series of sixteen acetylenic carbinols has been investigated for pharmacological activity. In contrast to the hypnotic activity generally found in this series, 3-octyne-2-ol and 3-nonyne-2-ol produced severe stimulation. The activity of certain of these compounds on various structures is presented.

WITHIN the past five years, two tertiary acetylenic carbinols have been utilized as sedative-hypnotics. Methylparafynol (1–5), introduced in 1951, and ethchlorovynol (6–8), introduced in 1955, are short-acting compounds which appear to have a low degree of toxicity.

Because of the continued interest in the acetylenic carbinols, the following compounds were obtained by this laboratory from Dr. John H. Wotiz, Department of Chemistry, University of Pittsburgh, for evaluation as possible hypnotics: 1. 6-heptyne-1-ol, 2. 5-heptyne-1-ol, 3. 4-heptyne-1-ol, 4. 3-heptyne-1-ol, 5. 2-heptyne-1-ol, 6.1-heptyne-3-ol, 7. 2-propyne-1-ol, 8. 2-methyl-3-nonyne-2-ol, 9. 3-octyne-2-ol, 10. 3-nonyne-2-ol, 11. 2-methyl-3-octyne-2-ol, 12. 3,5-dimethyl-1-hexyne-3-ol, 13. 3,6-dimethyl-1-heptyne-3-ol, 14. 3-isobutyl-5-methyl-hexyne-3-ol, 15. 3-methyl-1-nonyne-3-ol, 16. 4-ethyl-1-octyne-3-ol.

EXPERIMENTAL

Hypnotic Activity of the Experimental Compounds.—The HD_{50} for methylparafynol was obtained in the usual manner in order to determine the approximate dosage level for the experimental compounds. The effects of this control drug were later used to compare the efficacy of the sixteen acetylenic alcohols. The HD_{50} of methylparafynol administered via the intraperitoneal route to male albino rats (Sprague-Dawley, Wistar cross) weighing approximately 150 grams was 218 mg./Kg.

Doses of the experimental compounds ranging from 100 to 1,000 mg./Kg. were administered intraperitoneally to male albino rats using the following vehicles: 33 per cent v/v propylene glycol solution, compounds 1–6; 33 per cent v/v aqueous solution, compound 7; 10 per cent v/v suspension with one per cent Tween® 20 and one per cent Span® 80 in water, compounds 8–11; 10 per cent v/v suspension with 2.5 per cent Tween® 20 and one per cent Span® 80 in water, compounds 12–15; 10 per cent v/v suspension with one per cent Tween® 20 in water, compound 16.

The loss of righting reflex and other noticeable symptoms were recorded. Hypnotic activity usually occurred between 100 and 300 mg./Kg. The only compounds not exhibiting hypnotic activity were 3-octyne-2-ol, 3-nonyne-2-ol, and 2-propyne-1-ol. The most favorable activity was produced by compounds 11, 13, 14, and 16. However, since all of the depressant compounds appeared to be extremely toxic in comparison to methylparafynol, further studies seemed unwarranted.

Compounds 9 (3-octyne-2-ol) and 10 (3-nonyne-2-ol) appeared to possess a reverse type of activity, producing severe excitation, increased respiration and an increase in all visible secretions, with the tears being bloody and viscous. Compound 9 was the more active of the two. Because the results obtained by 3-octyne-2-ol in the intact rat were opposite to that usually seen with acetylenic alcohols, further studies were carried out in an attempt to determine the mechanism of action of this compound.

Pharmacology of 3-octyne-2-ol

Species Variation.—The compound, prepared as previously described, was administered via the ventral lymph sac to frogs and intraperitoneally to albino rats, mice, and rabbits. Doses ranging from 100 to 300 mg./Kg. produced depression in the frog. Doses of 150 to 500 mg./Kg. produced excitation and increased salivary and lacrimal secretions in rats, mice, and rabbits. The excitation in rats was blocked by administering 15 mg./Kg. of pentobarbital sodium intraperitoneally ten minutes prior to injecting 3-octyne-2-ol. The barbiturate did not antagonize the increased secretions. Atropine sulfate, 25 mg./Kg., administered intraperitoneally to the rats seven minutes prior to injecting the carbinol, blocked the increased in secretions.

Effect on the Isolated Rabbit Ileum.—Strips of rabbit ileum were suspended in a 70-ml. bath containing either oxygenated Locke-Ringer's solution, Sollmann-Rademaker's solution, or modified Locke's solution maintained at 38°. Doses between 5 and 10 mg. of 3-octyne-2-ol produced a marked decrease in the amplitude of the contractions. Five milligrams of the carbinol greatly depressed spasms induced by 3 mcg. of acetylcholine and blocked the activity of further doses of the spasmogenic agent (Fig. 1). A dose of 0.05 ml. of ethanol produced no apparent effect on Ach. induced spasms. Eighteen milligrams of 2-propyne-1-ol increased the tone of the intestinal strips approximately 50 per cent.

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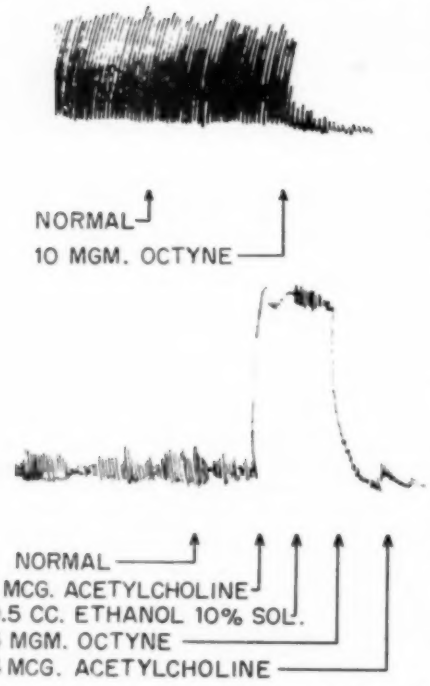

Fig. 1.—Effects of 3-octyne-2-ol on the isolated rabbit ileum.

Action on the Rat Uterus.—Rat uteri, approximately 2.5 cm. in length, were suspended in 70 ml. of oxygenated Locke-Ringer's solution maintained at 38°. A dose of 0.3 ml. of a 10 per cent emulsion of 3-octyne-2-ol decreased the height of the contractions and blocked the action of Pitocin.®

Rat Heart in situ.—Albino rats of either sex were anesthetized with 1.25 Gm./Kg. urethan intraperitoneally. The trachea was cannulated and artificial respiration administered, using a Phipps and Bird pulmotor valve. The rib cage was opened by a midline incision and a glass tube placed around the heart. The skin was sealed around the tube with wound clamps. The apex of the ventricle was attached to a muscle lever in the usual manner. Doses of 5 to 25 mg. (approximately 25 to 125 mg./Kg.) administered intraperitoneally greatly increased the amplitude of the contractions and slowed the rate slightly.

Rat Blood Pressure.—Albino rats of either sex were anesthetized with 1.1 Gm./Kg. urethan intraperitoneally. The left carotid artery was exposed and cannulated in the usual manner with a fine glass cannula. Ten per cent sodium citrate solution was used in the system as an anticoagulant. Intravenous injections of 10 to 35 mg./Kg. of 3-octyne-2-ol produced a slight pressor effect in normotensive rats. Doses of 15, 30, and 60 mg./Kg. of 2-propyne-1-ol did not noticeably alter the pressure, and 35 mg./Kg. of 3-methyl-1-nonyne-3-ol lowered the blood pressure between 40 and 50 per cent.

DISCUSSION

The results of the preliminary studies on this series of acetylenic carbinols indicated that the compounds were too toxic to warrant further studies.

The activity of 3-octyne-2-ol in rats deviated drastically from the expected hypnotic action of the acetylenic carbinols. The excitation and increased secretions indicated possible autonomic activity and/or central stimulation. The fact that 3-octyne-2-ol decreased intestinal and uterine activity, increased cardiac activity, produced a slight pressor effect and central excitation suggests probable sympathetic activity. The analog 2-propyne-1-ol increased intestinal tone and greatly increased secretions indicating possible parasympathetic stimulation.

The depression produced in frogs by 3-octyne-2-ol was similar to the results reported by Slater, *et al.* (9), with the convulsant diethylbutanediol. It is also interesting to note that the secondary alcohol, 3-octyne-2-ol, produced stimulation in rats, whereas the tertiary alcohol, 2-methyl-3-octyne-2-ol, was a very potent depressant.

The results of this investigation offers further proof that minute changes in chemical configuration may drastically affect physiological activity.

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Studies Relating to the Stability of Vitamin B_{12} in B-Complex Injectable Solutions*

By MILTON BLITZ, EDWARD EIGEN, and EPHRAIM GUNSBERG

Room temperature storage studies of various commercial B-complex injectables demonstrate the instability of vitamin B_{12} in these products. Experimental data are presented to show that vitamin B_{12} is unstable in the presence of thiamine and niacinamide at concentrations from 25 mg. to 100 mg. per cc. of each component in solutions at *p*H 4.25, but is relatively stable at lower concentrations. The mechanism of this decomposition is discussed.

RECENTLY, attention has been directed to the effect of thiamine and niacinamide on the stability of vitamin B_{12} (1, 2). The decomposition of vitamin B_{12} was shown to occur in several representative aqueous B-complex injectable products currently on the market during their normal shelf life (1). This finding suggested an investigation to determine the cause of the instability of vitamin B_{12} in this type of product. Confirmation of this instability was made by elevated temperature studies and the results showed that it was caused by the presence of thiamine and niacinamide (or niacin) in quantities normally found in B-complex injectables. Feller and Macek (2) also discussed the stability of vitamin B_{12} in similar solutions. Their work showed vitamin B_{12} to be unstable in solutions of thiamine and niacinamide stored at elevated temperatures but stable at room temperature. However, the study concerned itself with concentrations of thiamine and niacinamide (10 mg./cc.) which are relatively low for B-complex injectables, different *p*H, and storage testing conditions. A recent investigation of the problem by Ponci (3) confirmed the observation that thiamine and niacinamide in combination caused the destruction of vitamin B_{12} at elevated temperatures. Ponci showed that this occurred in solutions from *p*H 3.2 to *p*H 6.2 and he concluded that sterilization by heat was not feasible.

Since the elevated temperature data (1, 3) indicated that thiamine and niacinamide cause decomposition of vitamin B_{12}, it was necessary to investigate this problem further to determine what occurs after long term storage at room temperature. This is essential because in the last analysis room temperature studies more closely approximate the shelf life of the injectable product. Therefore, it is the purpose of this paper to present the results of the room temperature studies of various commercial parenterals and of various experiments investigating the effect of thiamine and niacinamide on vitamin B_{12} in aqueous solution.

PROCEDURE

The U. S. P. microbiological assay method (4) for vitamin B_{12} was used in these studies. Losses of 10% or less were considered insignificant, since the microbiological procedure is accurate to ±10%.

EXPERIMENTAL

The investigation of six currently available B-complex solutions is described in the present study. The composition of these injectables which were purchased at random on the retail market is shown in Table I. Table II presents the vitamin B_{12} assay results obtained on these products and indicates poor stability after storage at room temperature for nine months. In fact, in 5 out of the 6 representative products studied, the vitamin B_{12} values obtained were below the labeled potency when assayed immediately after purchase. Products B and E had lost more than 95% of the labeled vitamin B_{12} on initial assay. But these results are not entirely unexpected, since these products were bought on the open market and their age prior to receipt at the laboratory is not known. After storage at room temperature for three months losses ranged from 42.5 to greater than 99%. Increased losses were observed when the samples were stored at room temperature for nine months. Product C appears to have reached an equilibrium point and no further losses were found after three months. The greatest losses of vitamin B_{12} were obtained with the products having the largest concentrations of thiamine and niacinamide.

Previous experiments (1) with solutions exposed to elevated temperatures showed that the instability of vitamin B_{12} in aqueous B-complex parenterals was caused by the presence of thiamine and niacinamide in these products. The following experiment was devised to confirm this observation. Pure solutions containing various concentrations of thiamine, niacinamide, and vitamin B_{12} only, were prepared and adjusted to *p*H 4.25. After standing at room temperature for one year, the vitamin B_{12} potencies were measured microbiologically (Table

* Received from the Analytical and Control Laboratories, U. S. Vitamin Corp., Yonkers, N. Y. Published with the approval of Dr. Louis Freedman, Director of Research.

The authors wish to thank Dr. W. C. Gakenheimer, Merck & Co., Inc., for the 2-methyl-4-amino-5-hydroxymethylpyrimidine and the 4-methyl-5-β-hydroxyethylthiazole used in this study. We would also like to thank Mr. Reuben Levinton for his technical assistance.

TABLE I.—LABELED COMPOSITION OF B-COMPLEX PARENTERALS PURCHASED ON THE OPEN MARKET

Ingredient	Product A	B	C	D	E	F
Vitamin B_{12}, mcg./cc.	5	30	20	30	10	5
Thiamine, mg./cc.	25	25	10	50	90	50
Niacinamide, mg./cc.	100	100	50	100	90	50
Riboflavin, mg./cc.	2	2	3	2	4.5	1
Pyridoxine, mg./cc.	5	5	5	2	2.25	5
Panthenol, mg./cc.	5	5	5	2	...	5
Phenol, %	0.5	...	...	...	...	...
Benzyl alcohol, %	...	2	...	2	3	...
Gentisic acid ethanolamide, %	...	...	2.5	...	...	1
*p*H	3.66	3.54	4.09	4.07	4.12	4.25

III). It is evident from the results that thiamine and niacinamide have a profound effect on the stability of vitamin B_{12} and that the concentration is an important factor. A loss of 30% of the vitamin B_{12} potency was observed after storage for one year at room temperature in the presence of 25 mg. each of thiamine and niacinamide. A concentration of 50 mg. of each component caused a loss of 81% after six months at room temperature and greater than 99% after one year. At the 75 mg. and 100 mg. levels of thiamine and niacinamide, greater than 99% losses were noted after only six months at room temperature. These room temperature results agree with those obtained at 45° where significant losses were observed after only five days at the same levels reported here. Although there is some loss of vitamin B_{12} at the lower concentrations, there is still a fairly good percentage of vitamin B_{12} left after one year, i. e., 70% retention at the 25 mg. level of thiamine and niacinamide. However, above this concentration there appears to be a definite break and the destruction of vitamin B_{12} increases very rapidly so that at the 50 mg. to 100 mg. of thiamine and niacinamide concentration practically all the vitamin B_{12} is destroyed. The results indicating the relative stability of vitamin B_{12} at the lower thiamine and niacinamide concentrations confirm work reported by other investigators (2). These data also explain why product C in Table I is the most stable one because this injectable only contains 10 mg./cc. of thiamine. It must be emphasized that the results of these tests are of practical significance because all the other commercially available B-complex injectables studied are high in thiamine and niacinamide content. Since large losses of vitamin B_{12} in these types of products were found to be due to the presence of both thiamine and niacinamide, it was important to determine if the factor responsible for the destruction of vitamin B_{12} is formed by an interaction between the two vitamins. Therefore, the following experiment was prepared. A solution containing 50 mg./cc. of thiamine and niacinamide at *p*H 4.25 was heated in a sealed vial for four hours at 100°. After cooling the solution to room temperature crystalline vitamin B_{12} was added. The results obtained after storage at room temperature and at 45° are shown in Table IV. A 20% loss of vitamin B_{12} was observed after only two days at room temperature. The destruction at 45° was practically 100% which

TABLE II.—THE STABILITY OF VITAMIN B_{12} IN VARIOUS B-COMPLEX PARENTERALS STORED AT ROOM TEMPERATURE

B-Complex Injectable	Loss, %, of Vitamin B_{12}[a] After Storage at Room Temperature for— Initial	3 Months	6 Months	9 Months
A	10	52	...	76
B	>95	>99	...	>99
C	20	42.5	41.5	42.5
D	46.7	88.3	...	91.7
E	>95	...	...	...
F	None	>94	>95	>98

[a] All the samples were analyzed for vitamin B_{12} immediately after purchase on the open market. The per cent loss was expressed as per cent of labeled claim.

TABLE III.—EFFECT OF VARYING AMOUNTS OF BOTH THIAMINE AND NIACINAMIDE ON A SOLUTION CONTAINING 5 MCG./CC. OF VITAMIN B_{12} AFTER STORAGE AT ROOM TEMPERATURE

Concentration of Both Thiamine and Niacinamide,[a] mg./cc.	Loss, %, of Vitamin B_{12} After Storage at Room Temperature for 6 Months	12 Months
1	None	None
10	None	17.4
25	None	30.0
50	81.0	>99
75	>99	>99
100	>99	>99

[a] Each solution contained equal amounts of thiamine hydrochloride and niacinamide and was adjusted to *p*H 4.25.

TABLE IV.—THE EFFECT OF PREHEATING A SOLUTION OF THIAMINE HYDROCHLORIDE AND NIACINAMIDE ON THE STABILITY OF A SOLUTION CONTAINING 5 MCG./CC. OF VITAMIN B_{12}[a]

Storage Conditions	Loss of Vitamin B_{12}, %
2 days at room temperature	20.0
1 week at room temperature	30.0
1 month at room temperature	62.4
3 months at room temperature	72.0
6 months at room temperature	81.6
4 days at 45°	97.6
1 week at 45°	>99
3 weeks at 45°	>99

[a] The solution contained 50 mg./cc. each of thiamine hydrochloride and niacinamide at *p*H 4.25 and was heated for four hours at 100° prior to the addition of vitamin B_{12}.

was much greater than that observed in previously unheated solutions of thiamine and niacinamide. Since vitamin B_{12} has been found to be stable in solutions in the presence of niacinamide in concentrations up to 100 mg./cc., it seemed obvious that a decomposition product of thiamine was responsible for the destruction. This has been suggested in a previous report (2). This predication was confirmed by the results obtained when vitamin B_{12} was added to a two year old solution of thiamine hydrochloride (Table V). This experiment shows that a partially decomposed thiamine solution assaying 90% of its original potency has a destructive effect on vitamin B_{12}. Although large losses of vitamin B_{12} were obtained with the aged solution, none was observed with a freshly prepared solution of thiamine, used as a control.

TABLE V.—THE EFFECT OF AGED AND FRESH THIAMINE SOLUTIONS ON THE STABILITY OF VITAMIN B_{12}[a]

Storage Conditions	Loss, %, of Vitamin B_{12} in the Presence of Aged Thiamine Solution	Loss, %, of Vitamin B_{12} in the Presence of Fresh Thiamine Solution
3 days at 45°	51.6	None
1 week at 45°	52.8	None
3 weeks at 45°	66.4	None
3 weeks at room temperature	20.0	None
3 months at room temperature	40.0	None

[a] Concentrations of test solutions were: thiamine hydrochloride, 50 mg./cc., and vitamin B_{12}, 5.0 mcg./cc.; pH 4.25.

A previous report (2) demonstrated that the thiazole portion of thiamine (equivalent to 10 mg. of thiamine) had a destructive effect on vitamin B_{12} at elevated temperatures. Since the parenteral products have much higher potencies, an experiment was conducted to determine the effects of the thiazole and pyrimidine nuclei equivalent to the amount present in 50 mg. of thiamine hydrochloride and also with the addition of niacinamide (Table VI). The data indicate that under conditions of the test, vitamin B_{12} is destroyed in the presence of the thiazole moiety. The losses of vitamin B_{12} observed in all test solutions where the thiazole moiety was present alone and with added niacinamide were slightly lower than those obtained in the presence of thiamine and niacinamide. This is most evident after 3 days at 45° and 3 weeks at 45°. No significant difference in destruction was observed in the values obtained after 1 week at 45°. Decomposition is so great at 100° that no distinction can be made among the three solutions. These results indicate that the possible formation of the thiazole fraction from thiamine is not responsible for all the destruction of vitamin B_{12} but suggest that another factor exists which causes further degradation of the vitamin.

Since decomposed thiamine had been established as a factor destroying vitamin B_{12} potency, attempts were made to determine the function of niacinamide in the reaction. This question had arisen from the results obtained in the experiments reported in Tables III and IV, and the elevated temperature studies reported previously (1). These studies show that the greatest destruction of vitamin B_{12} occurs only in the presence of both thiamine and niacinamide. Niacinamide is known to cause the deterioration of thiamine in solution (5). In view of the now known effect of decomposition products of thiamine on vitamin B_{12}, it appears that niacinamide acts as the agent for the release of a substance from thiamine which is detrimental to vitamin B_{12}. To test this thesis, niacinamide was replaced by its parent compound, pyridine, in this system (Table VII). No degradation of vitamin B_{12} was observed after three weeks at 45° and six months at room

TABLE VII.—EFFECT OF SUBSTITUTING PYRIDINE FOR NIACINAMIDE ON THE STABILITY OF VITAMIN B_{12} IN THE PRESENCE OF THIAMINE[a]

Storage Conditions	Loss, %, of Vitamin B_{12} in the presence of Thiamine plus Pyridine	Loss, %, of Vitamin B_{12} in the presence of Pyridine Only
4 hours at 100°	>99	23.2
4 days at 45°	70.0	None
1 week at 45°	>99	None
3 weeks at 45°	>99	None
1 month at room temperature	25.0	None
3 months at room temperature	22.4	None
6 months at room temperature	>99	12.0
9 months at room temperature	>99	15.0
12 months at room temperature	>99	13.4

[a] Concentrations used were: thiamine hydrochloride, 50 mg., pyridine, 50 mg., and vitamin B_{12}, 5 mcg./cc.; pH 4.25.

TABLE VI.—THE EFFECT OF THE THIAZOLE AND PYRIMIDINE FRACTIONS OF THIAMINE ON THE STABILITY OF VITAMIN B_{12}

Test Solution[a]	Loss, %, of Vitamin B_{12} After Treatment for 4 hours at 100°	3 days at 45°	1 week at 45°	3 weeks at 45°
Vitamin B_{12} + pyrimidine moiety	None	None	None	None
Vitamin B_{12} + thiazole moiety	>99	36.6	75.0	78.4
Vitamin B_{12} + pyrimidine and thiazole moieties	>99	33.4	69.0	81.0
Vitamin B_{12} + pyrimidine moiety + niacinamide	26.6	None	None	None
Vitamin B_{12} + thiazole moiety + niacinamide	>99	40.0	69.0	86.6
Vitamin B_{12} + pyrimidine and thiazole moieties + niacinamide	>99	40.0	68.0	81.4
Vitamin B_{12} + thiamine	36.6	None	None	None
Vitamin B_{12} + thiamine and niacinamide	>99	56.6	79.2	>99

[a] Quantities used in these experiments were: crystalline vitamin B_{12}, 5 mcg./cc.; 2-methyl-4-amino-5-hydroxymethyl pyrimidine, 29 mg./cc.; 4-methyl-5-β-hydroxyethyl thiazole, 21 mg./cc.; thiamine hydrochloride, 50 mg./cc.; niacinamide, 50 mg./cc. All solutions were adjusted to pH 4.25.

temperature in the presence of pyridine alone. In the presence of both pyridine and thiamine, however, destruction of the vitamin was complete after six months at room temperature and only 1 week at 45°. At 100° for four hours destruction was complete in the presence of the mixture, whereas, only 23.2% of vitamin B_{12} was lost with pyridine alone. It appears, therefore, that niacinamide or the pyridine nucleus, acts upon thiamine to form a compound that is incompatible with vitamin B_{12}.

Vitamin B_{12} has been found stable in low potency elixirs (2). Our studies on all oral products (not containing ascorbic acid) and elixir type products always have shown good vitamin B_{12} stability. No difficulty should be encountered with these products, since the thiamine and niacinamide content is relatively low.

SUMMARY

1. Studies of certain commercially available B-complex injectable products show instability of vitamin B_{12} after storage at room temperature for nine months.

2. This instability has been shown to be due to the presence of thiamine and niacinamide in combination at concentrations from 25 mg. to 100 mg./cc. of each in an aqueous solution adjusted to *p*H 4.25.

3. Destruction of vitamin B_{12} is diminished at decreasing concentrations of thiamine.

4. Both partially decomposed thiamine and the thiazole moiety of thiamine destroy vitamin B_{12} in aqueous solutions at *p*H 4.25 in the absence of niacinamide.

5. Pyridine can substitute for niacinamide in the destruction of vitamin B_{12} in the presence of thiamine.

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A Comparison of the Stability of Cyanocobalamin and Its Analogs in Ascorbate Solution*

By HASTINGS H. HUTCHINS, PATRICIA J. CRAVIOTO, and THOMAS J. MACEK

The stability of eight vitamin B_{12} products in 1% ascorbic acid solution in 1 molar acetate buffer at *p*H 4.0 has been studied during storage at constant temperatures. Cyanocobalamin (crystalline vitamin B_{12}) was shown to be markedly more stable than cyanide-free B_{12} analogs in ascorbate solutions. Vitamin B_{12} concentrates containing mixtures of cyanocobalamin and noncyano analogs were found to be less stable in ascorbate solution than concentrates containing cyanocobalamin exclusively. The stability of vitamin B_{12} concentrates thus appears to be related directly to the concentration of cyanocobalamin present in the concentrate.

CRYSTALLINE vitamin B_{12}, a coordination complex involving a cyano group and organically bound cobalt, gives rise to a series of cobalamins simply by replacement of the cyano substituent with certain other anions (1, 2). The cyanide-free analogs, originally designated by subgroupings B_{12a}, B_{12b}, etc., are now named after the anion-containing moiety, *viz.*, chlorocobalamin,[1] hydroxycobalamin, nitrocobalamin, thiocyanatocobalamin, etc. (3). All exhibit similar ultraviolet absorption spectra and are microbiologically active for *Lactobacillus lactis* Dorner and *Lactobacillus leichmannii* (2). These characteristics make it possible to compare stability of the vitamin B_{12} analogs with cyanocobalamin in aqueous solution.

A difference in stability to ascorbic acid was previously noted between cyanocobalamin and hydroxycobalamin (4–8). This reaction with ascorbic acid therefore was considered useful for measuring differences in stability between cyanocobalamin and various other analogs in the present study. For this purpose analogs in both

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[1] The term "cyanocobalamin" when used to describe the chemical composition of the vitamin B_{12} analog in concentrates is used in a chemical sense and not according to U. S. P. definition. Cyanocobalamin, as found in concentrates, is commonly amorphous; cyanocobalamin as defined in the U. S. P. XV is a pure crystalline compound.

the pure form and as various commercially-available concentrates of vitamin B_{12} were used. The presence of analogs other than cyanocobalamin in vitamin B_{12} concentrates has been suggested as a significant factor in the instability of such concentrates in pharmaceutical mixtures. Several authors have reported better stability of vitamin B_{12} in aqueous solution as a function of increasing concentration of cyanocobalamin in the vitamin B_{12} sample (4, 9).

In order to establish the cyanocobalamin content of the vitamin B_{12} concentrates used in this study the concentrates were assayed by the procedure of Bacher, *et al.* (10). Determinations made on the four samples indicated that one concentrate contained more than 99% cyanocobalamin; three others varied in cyanocobalamin content between 20 and 50%.

EXPERIMENTAL

Solutions of the vitamin B_{12} analogs were prepared at a concentration of 25 mcg./ml. in 1 molar aqueous sodium acetate-acetic acid buffer at *p*H 4.0. The solutions were assayed by the U. S. P. XIV method using *Lactobacillus leichmannii*. Aqueous solutions of cyanocobalamin were previously reported to be stable in this *p*H range (6). After removing the initial sample for assay, crystalline ascorbic acid (10 mg./ml.) was added to each of the solutions and a second sample was removed for assay for vitamin B_{12}. The samples were then stored and assayed frequently by the U. S. P. XIV method for total vitamin B_{12} activity.

The solutions of cyanocobalamin and the four vitamin B_{12} concentrates were stored at 30° in a constant temperature bath for various periods of time up to 6 days. Solutions containing nitrocobalamin, chlorocobalamin, and thiocyanatocobalamin were stored at a slightly lower temperature (25°) for three hours. During this time more than 92% of the vitamin B_{12} activity was destroyed in each of the latter samples. The results were checked in all cases both by duplication of the individual assays on separate days and by repetition of the entire experiment. The decomposition rate remained essentially the same in all experiments. During the test period no change in *p*H was observed in any of the samples.

Data indicating the stability of the various test solutions are summarized in Table I.

DISCUSSION

The data summarized in Table I indicate stability of eight vitamin B_{12} products in 1% ascorbic acid solution buffered with 1 molar sodium acetate-acetic acid at *p*H 4.0. This *p*H was used since it generally provides maximum stability for solutions of vitamin B_{12} alone and in the presence of other B vitamins. In a separate test, incidentally, it was observed that the rate of decomposition of thiocyanatocobalamin and nitrocobalamin was the same in unbuffered solution at *p*H 2.75 as is reported in buffered solution at *p*H 4.0.

Temperature was observed to have a noticeable effect on vitamin B_{12} analog stability. At 30° de-

TABLE I.—STABILITY OF VITAMIN B_{12} AND VITAMIN B_{12} ANALOGS IN ASCORBATE SOLUTION

	Micrograms per ml. (L. L.)							
	Stored at 30°					Stored at 25°		
Test Period	Cyanocobalamin, U. S. P.	Vitamin B_{12} Concentrate, Sample A	Vitamin B_{12} Concentrate, Sample B	Vitamin B_{12} Concentrate, Sample C	Vitamin B_{12} Concentrate, Sample D	Chlorocobalamin	Nitrocobalamin	Thiocyanatocobalamin
Cyanocobalamin content	97.7% (dry basis)	100%	20%	50%	50%	4.5%	Less than 2%	4.5% or less
Initial assay	29.6	29.8	24.5	26.2	28.8	16.8	24.0	24.0
Ascorbic acid added								
Initial assay	28.1	27.4	12.2	24.4	26.8	3.7	21.2	5.3
15 min.	..	..	..	..	..	1.19	6.3	2.0
30 min.	..	..	7.9	..	..	0.87	3.0	1.8
1 hr.	..	..	7.5	..	..	0.72	0.31	2.0
2 hr.	23.6	27.5	6.4	16.5	13.8	..	..	..
3 hr.	..	..	..	..	22.4	0.68	0.22	1.7
4 hr.	..	26.4	4.8	19.5	17.5	..	..	..
6 hr.	22.3	26.4	4.9	14.5	14.8	..	..	..
8 hr.	22.5	27.1	3.8	18.7	16.7	..	..	..
12 hr.	18.1	27.7	3.7	14.8	16.1	..	..	..
16 hr.	15.6	24.5	3.3	12.3	13.0	..	..	..
20 hr.	..	25.1	2.3	11.1	12.5	..	..	..
24 hr.	23.3	21.8	2.4	10.2	11.1	..	..	..
2 days	17.9	24.5	0.49	5.7	9.5	..	..	..
3 days	15.8	..	0.44	..	..	..	..	..
4 days	19.0	23.5	..	6.9	10.6	..	..	..
5 days	13.2	26.2	0.44	..	..	..	..	..
6 days	16.7	17.6	0.37	4.0	7.6	..	..	..

composition of the nitro-, chloro-, and thiocyanato-analogs was sufficiently rapid to cause difficulty in sampling. For this reason it was found better to study the stability of the analogs at a slightly lower temperature, i.e., 25° instead of 30°. Vitamin B_{12} decomposition was observed to be completely arrested during storage of samples at −10°. This observation enabled the authors to store samples and reassay them for confirmation of results on subsequent days.

Data given in Table I indicate that vitamin B_{12} concentrate stability in ascorbic acid solution is greater with increasing concentration of cyanocobalamin present. The presence of noncyano analogs in vitamin B_{12} concentrates resulted in a correspondingly greater vitamin B_{12} instability. Thus vitamin B_{12} concentrate (Sample B) containing only 20% cyanocobalamin proved to be less stable than B_{12} concentrates containing 50% cyanocobalamin (Samples C and D). The latter samples also were less stable than vitamin B_{12} concentrate containing all cyanocobalamin (Sample A).

The effect of ascorbic acid on stability of the different forms of vitamin B_{12} was quite pronounced. Cyanocobalamin and cyanocobalamin-containing B_{12} concentrates were found to be most stable to ascorbic acid. Noncyanocobalamins in pure form on the other hand were quite unstable. Marked decomposition even occurred in these solutions during the brief interval between addition of ascorbic acid and immediate sampling for initial assay.

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The Effect of Prednisolone and Prednisone on the Oxygen Uptake of Inflamed Tissue*

By JOHN G. McDONALD and HAROLD C. HEIM†

Inflammation was induced in rats by injecting air followed by a solution of croton oil in corn oil under the skin of the backs of the animals. Forty-eight hours after the injection of the croton oil some of the animals received 5.0 mg. of prednisone or prednisolone by injection into the inflamed area. Forty-eight hours after injection of the steroids the animals were sacrificed, the inflamed tissue was removed, homogenized, and the Q_{O_2} determined by conventional manometric techniques. Some of the animals received no steroids and served as the controls. It was found that tissue from the animals treated with the steroids showed a higher Q_{O_2} than did tissue from animals which received no steroids. The results were found to be statistically significant.

ALTHOUGH cortisone and hydrocortisone are widely used in the clinical management of certain inflammatory conditions, the mechanisms by which these drugs exert their antiphlogistic effects are not well understood. Recently the Δ^1 analogs of cortisone and hydrocortisone have been introduced as therapeutic agents under the names of prednisone and prednisolone, respectively, and clinical reports indicate that they are powerful antiphlogistic agents (1).

In a previous study (2) it was observed that the oxygen uptake of homogenates prepared from inflamed tissue was increased if the inflamed tissue had been previously treated with hydrocortisone. This effect was not observed with certain steroids which do not exhibit antiphlogistic activity. Because prednisone, prednisolone, and hydrocortisone all elicit similar therapeutic effects, it appeared of interest to determine the effects of prednisone and prednisolone on the oxygen uptake of homogenates prepared from inflamed tissue which had previously been treated with these

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drugs. It was felt that studies involving the effects of glucocorticoids upon cellular processes might conceivably lead to the elaboration of mechanisms by which steroid therapy alleviates the inflammatory process.

EXPERIMENTAL

Inflamed tissue was produced in adult Sprague-Dawley rats by a method which is very similar to that described by Selye (3). Ten cubic centimeters of air was injected under the skin of the backs of the animals and into the pouch thus formed 0.5 cc. of 5 per cent croton oil in corn oil was injected. Forty-eight hours later some of the animals received 5.0 mg. of prednisone or prednisolone in aqueous suspension by injection into the pouch. The remaining animals were used as the controls. Ninety-six hours after the injection of the croton oil the animals were sacrificed and the tissue comprising the pouch was removed. This was accomplished by slitting the pouch and stripping the inflamed tissue from the skin and subcutaneous muscle. The tissue was washed by immersing it in distilled water after which it was quickly blotted dry on filter paper and then weighed. It was then homogenized in distilled water in a glass homogenizer which was kept immersed in ice during the homogenization process. The homogenates were prepared so that 0.5 cc. contained 50 mg. of fresh tissue.

For the measurement of oxygen uptake 0.5 cc. of homogenate was added to the main compartment of Warburg vessels containing 0.3 cc. of 0.01 *M* potassium adenylate and 2.0 cc. of 0.1 *M* potassium phosphate buffer, *p*H 7.4. The center wells contained 0.2 cc. of 10 per cent KOH together with a pleated strip of filter paper. After a ten-minute equilibration period, the manometers were closed and the oxygen consumption was measured after sixty minutes had elapsed. The temperature of the bath was 37° and the gas phase was air.

In order to report the oxygen consumption as the Q_{O_2} value (mm.^{3} O_2 consumed/mg. dry tissue/hour), aliquots of the homogenates were measured into small tared watch glasses which then were placed in an oven at 110°. After two hours the dishes were cooled and weighed, the weight thus obtained being used to calculate the dry weight of tissue present in 0.5 cc. of homogenate.

RESULTS AND DISCUSSION

The values for Q_{O_2} are shown in Table I. The results indicate that the Q_{O_2} is higher with the tissue from the pouches into which prednisone or prednisolone had been injected forty-eight hours prior to the experiment. The results expressed in the table were subjected to statistical analysis and were found to be significant at the 99 per cent level.

It has been reported (4) that prednisone exhibits about four times the antirheumatic potency of hydrocortisone and that the potencies of prednisone and prednisolone are about equal. With the methods used in this study we were unable to show any significant difference in the effect exerted on the Q_{O_2} values by either prednisone or prednisolone,

TABLE I.—RESULTS

	No. Animals	Mean Q_{O_2}	Standard Error[a]
Controls	29	3.32	0.19
Prednisone	18	4.27	0.23
Prednisolone	17	4.57	0.28

[a] $e = \sqrt{\Sigma d^2/n(n-1)}$.

nor were the results markedly different from those obtained with hydrocortisone as previously reported (2).

That the effect of these drugs on the Q_{O_2} was not elicited *in vitro* was shown by the fact that addition of prednisolone or prednisone to the homogenates in the Warburg vessels did not significantly increase the Q_{O_2}.

In the course of this study it was found that the degree of inflammation seemed to influence the magnitude of the Q_{O_2}. In most instances, forty-eight hours after the injection of the steroids the exudate had disappeared from the pouch but considerable highly inflamed tissue remained. In a few animals, however, the inflammation had completely disappeared forty-eight hours after the injection of the steroids and the tissue from the pouches on these animals had Q_{O_2} values lower than those obtained when evidence of inflammation was still present. This would appear to indicate that the effects elicited by the steroids on the Q_{O_2} values occur only when the inflammatory process is flourishing. Some evidence has appeared (5, 6) which indicates that the steroid therapy of rheumatic heart disease is most effective if performed early in the course of the disease. It seems plausible to assume, from such evidence, that the flourishing inflammatory process encountered in the acute manifestations of rheumatic fever might be most susceptible to the antiphlogistic effects of the corticoids. Such an assumption would find some degree of correlation with the results reported herein.

SUMMARY

Inflammation was induced subcutaneously in rats and forty-eight hours later either prednisone or prednisolone was injected into the inflamed area. Ninety-six hours later the tissue was removed from the pouch, homogenized, and the Q_{O_2} determined. The Q_{O_2} values with the tissues treated with the steroids were significantly higher than those obtained with tissue from the control animals.

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Formation of Molecular Complexes by Some Water-Soluble Amides II.

Effect of Decreasing Water Solubility on Degree of Complex Formation*

By H. B. KOSTENBAUDER† and T. HIGUCHI

The formation of molecular complexes in aqueous solution was generally enhanced, as expected, by the "squeezing out" effect of water on complexing agents rendered less water-soluble by structural modifications. The stereochemical effect, however, appears to impose limitations on these modifications. Substitution of ethyl for methyl in several dialkyl amides resulted, in most cases, in an apparent increase in complex formation and marked decrease in the solubility of the complex formed.

THE MOLECULAR COMPLEXES formed by some highly water-soluble amides of dimethylamine, as noted in a previous study (1), exhibited relatively low apparent stability constants. The present investigation was undertaken to determine the effect of employing the corresponding amides of diethylamine in an attempt to obtain a less water-soluble complexing agent. If the complexing agent is less water-soluble the "squeezing out" effect of water (2) on the relatively hydrophobic complexing agent and drug should enhance the complex formation in aqueous solution. This expectation was borne out, in general, by experimental findings.

The complexing agents used in the comparison were N,N,N′,N′-tetraethylisophthalamide, N,N,N′,N′-tetraethylterephthalamide, and N,N,N′,N′-tetraethylfumaramide. Observations were made on the complexing tendencies of these amides with *p*-hydroxybenzoic acid, salicylic acid, and chloramphenicol, utilizing the solubility method described in previous studies (1, 3).

EXPERIMENTAL OBSERVATIONS

Nature of Species in Aqueous Solutions of the Amides.—The partition isotherms for the several amides employed in the complexing studies are illustrated in Figs. 1 and 2. The linearity of the partition isotherms for N,N,N′,N′-tetraethylfumaramide and N,N,N′,N′-tetraethylterephthalamide suggests that the same species exist in both the aqueous and the organic phases and in all probability are monomeric. The partition isotherm for N,N,N′,N′-tetraethylisophthalamide deviates slightly from linearity but over the concentration ranges employed in this study it is possible to assume that for practical purposes the species in the aqueous and organic phases are identical and probably are monomeric.

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Interactions with p-Hydroxybenzoic Acid.—The solubility of *p*-hydroxybenzoic acid in water at 30° in the presence of varying concentrations of N,N,N′,N′-tetraethylfumaramide and N,N,N′,N′-tetraethylisophthalamide is illustrated in Fig. 3.

The addition of N,N,N′,N′-tetraethylfumaramide causes an initial increase in the solubility of *p*-hydroxybenzoic acid. The linearity of this portion of the curve indicates that the principal complex being formed has a first order dependency with respect to the amide concentration and is probably of the type

$$\text{Amide} + n\ \text{Acid} \rightleftharpoons \text{Amide-(Acid)}_n$$

The true stoichiometric ratio of acid to amide in the soluble complex cannot be determined by solubility studies, but for the purpose of obtaining a comparison of complexing tendencies the ratio can be considered to be effectively 1:1 and apparent stability

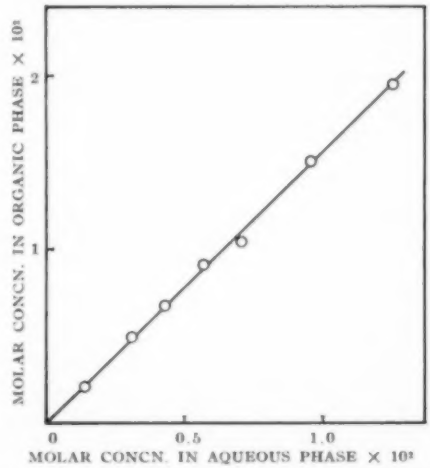

Fig. 1.—Partition isotherm for N,N,N′,N′-tetraethylterephthalamide, 30°. Organic phase: 10% v/v chloroform in iso-octane. Aqueous phase: distilled water.

TABLE I.—COMPARISON OF SOLUBILITY AND COMPLEXING TENDENCY OF SEVERAL AMIDES AT 30°

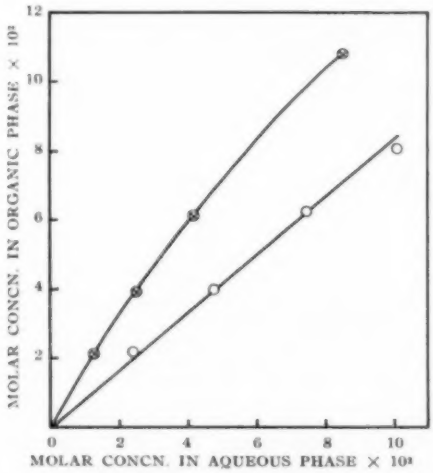

Fig. 2.—Partition isotherms, 30°. Organic phase: 10% v/v chloroform in iso-octane. Aqueous phase: distilled water. ⊗—N,N,N′,N′-Tetraethylisophthalamide. ○—N,N,N′,N′-Tetraethylfumaramide.

constants are calculated on this basis. These values are included in Table I.

Analysis of the phase diagram for the N,N,N′,N′-tetraethylfumaramide system, as described in previous studies (1, 3), indicates precipitation of a relatively insoluble complex consisting of a large number of acid molecules to each amide molecule in the complex. Isolation and subsequent chemical analysis of the material precipitated along the downcurve gave a ratio of 6.0 acid molecules to each amide molecule.

The addition of N,N,N′,N′-tetraethylisophthalamide did not increase the solubility of *p*-hydroxybenzoic acid but caused immediate precipitation of a practically insoluble complex. The length of the plateau region and chemical analysis of the precipitate obtained along the downcurve indicated that the complex being precipitated had a molecular ratio of 1.0 acid group to each amide. The slope of the downcurve is −0.9 which corresponds to the precipitation of 0.9 mole of complex on the addition of 1.0 mole of amide. The apparent stability constant could not be calculated for this interaction due to the extreme insolubility of the complex being formed.

The interaction of N,N,N′,N′-tetraethylterephthalamide with *p*-hydroxybenzoic acid did not increase the solubility of the acid, but gave a plateau region of the approximate length corresponding to a 1:1 interaction. Accurate determinations became impossible around the termination of the plateau region due to supersaturation and resulting inability to reproduce results.

Interactions with Salicylic Acid.—Phase diagrams showing the influence of N,N,N′,N′-tetraethylfumaramide, N,N,N′,N′-tetraethylisophthalamide, and N,N,N′,N′-tetraethylterephthalamide on the solubility of salicylic acid in water at 30° are presented in Fig. 4.

The plateau region in the phase diagram for the interaction of salicylic acid with N,N,N′,N′-tetraethylfumaramide indicates that the complex being precipitated had a ratio of 3.0 acid molecules to each amide molecule. Chemical analysis of the material

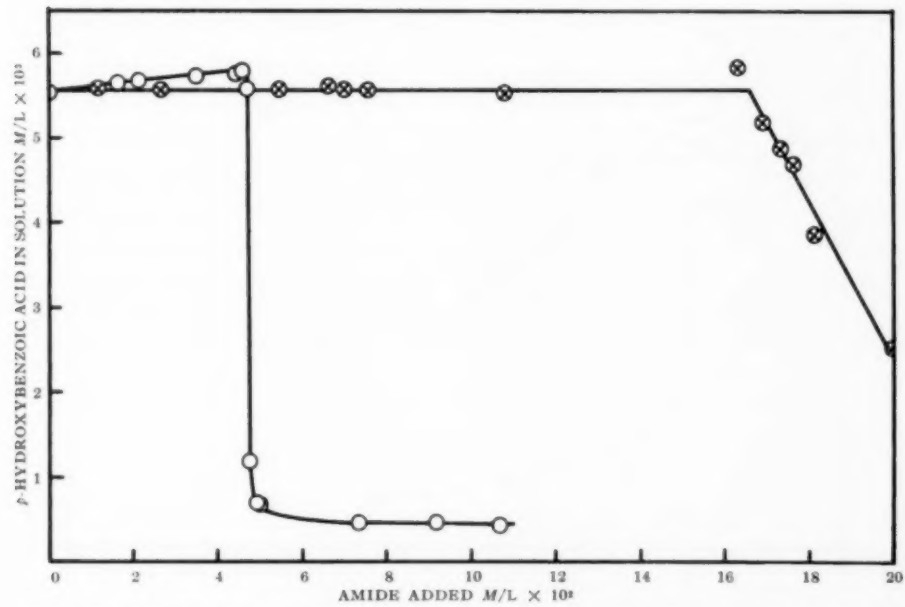

Fig. 3.—Solubility of *p*-hydroxybenzoic acid in water at 30° in the presence of: ○—N,N,N′,N′-tetraethylfumaramide; ⊗—N,N,N′,N′-tetraethylisophthalamide.

TABLE I.—COMPARISON OF SOLUBILITY AND COMPLEXING TENDENCY OF SEVERAL AMIDES AT 30°

Amide	Solubility of Amide *M*/L.	Apparent Stability Constants for 1:1 Interaction in Aqueous Solution		
		p-Hydroxybenzoic Acid	Salicylic Acid	Chloramphenicol
N,N,N′,N′-tetramethylisophthalamide	3.07	5.7	3.5	2.7
N,N,N′,N′-tetraethylisophthalamide	0.72	..	4.5	3.5
N,N,N′,N′-tetramethylterephthalamide	1.84	3.7	2.6	2.4
N,N,N′,N′-tetraethylterephthalamide	0.02	..	..	3.5
N,N,N′,N′-tetramethylfumaramide	1.73	5.9	4.7	..
N,N,N′,N′-tetraethylfumaramide	0.69	1.1	4.5	..

being precipitated along the downcurve gave a ratio of 2.2 acid molecules to one amide molecule. The apparent stability constant calculated on the assumption of a 1:1 interaction for that portion of the curve showing increasing solubility of salicylic acid with increasing amide concentration is noted in Table I.

Analysis of the phase diagram for the N,N,N′,N′-tetraethylisophthalamide-salicylic acid system gave a molecular ratio of 1.9 acid to one amide. The insoluble complex which formed in this interaction was an oil.

Addition of N,N,N′,N′-tetraethylterephthalamide did not increase the solubility of salicylic acid but resulted in precipitation of an extremely insoluble complex. Analysis of the phase diagram for this interaction gave a ratio of 2.0 acid molecules to each amide. Chemical analysis of the precipitate obtained along the downcurve also gave a molecular ratio of 2.0 acid to one amide.

Interactions with Chloramphenicol.—The solubility of chloramphenicol in water at 30° was studied as a function of the concentration of N,N,N′,N′-tetraethylisophthalamide and N,N,N′,N′-tetraethylterephthalamide. These data are presented in Fig. 5, and the apparent stability constants calculated on the basis of a 1:1 interaction are included in Table I.

The plateau occurring at the point S in the interaction of chloramphenicol with N,N,N′,N′-tetraethylterephthalamide is not due to precipitation of complex, but to saturation of the solution with respect to the complexing agent itself.

GENERAL DISCUSSION

As shown in Table I, the amides of diethylamine are less water-soluble than the corresponding amides of dimethylamine; and, with the exception of N,N,N′,N′-tetraethylfumaramide, complex formation appeared to be enhanced by the decrease in solubility. The water solubility of the tetraethylfumaramide is considerably less than that of the tetramethylfumaramide, but the steric effect due

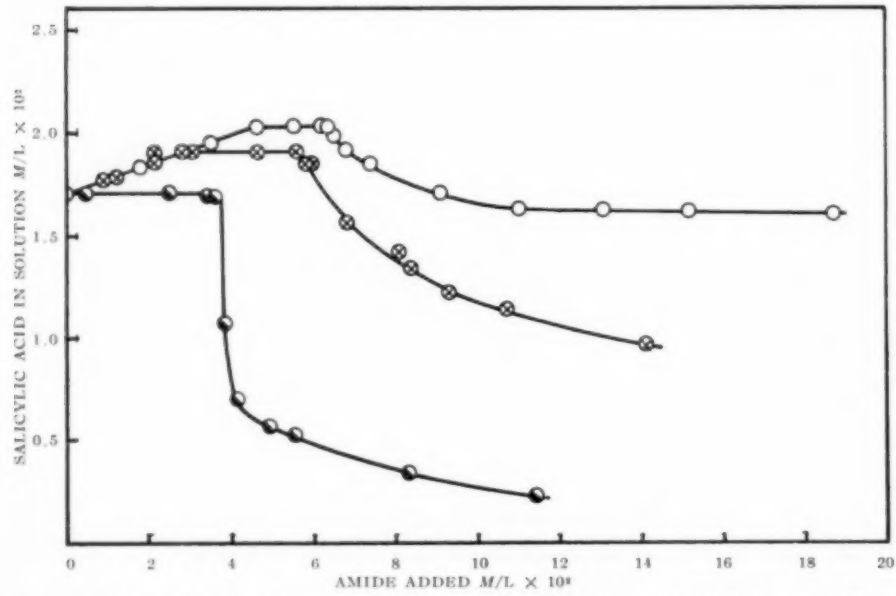

Fig. 4.—Solubility of salicylic acid in water at 30° in the presence of: ○—N,N,N′,N′-tetraethylfumaramide; ⊗—N,N,N′,N′-tetraethylisophthalamide; ◖—N,N,N′,N′-tetraethylterephthalamide.

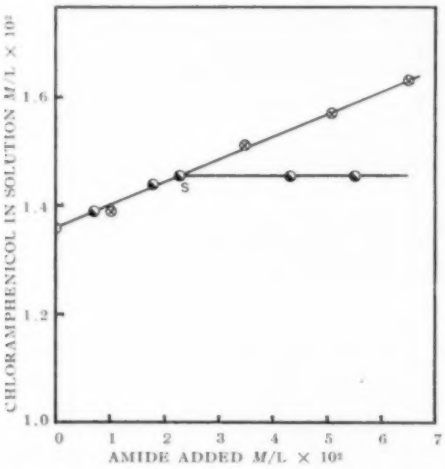

Fig. 5.—Solubility of chloramphenicol in water at 30° in the presence of: ⊘—N,N,N′,N′-tetraethylisophthalamide; ◖—N,N,N,′N,′-tetraethylterephthalamide. The point S corresponds to saturtion with respect to N,N,N′,N′-tetraethylterephthalamide.

to the larger ethyl groups probably was responsible for the decrease in degree of complex formation with this amide. If the study were extended to amides of longer chain amines it is probable that a limiting chain length beyond which the steric effect becomes more important than the increase in complexing tendency due to decreased water solubility would also have been observed for the isophthalamides and terephthalamides. This limiting chain length may be a function not only of the nature of the amide itself, but also of the drug which is to be complexed.

The results of these studies are in accord with previously proposed theories (2) in which the squeezing out effect of water on the lipophilic portions of the molecules was considered to be an important factor in the formation of molecular complexes in aqueous solution.

EXPERIMENTAL PROCEDURE

Preparation of Amides.—The amides were prepared by treating solutions of the respective acid chlorides in anhydrous ether with diethylamine. The N,N,N′,N′-tetraethylfumaramide was isolated and purified in a manner analogous to that described for the amides of dimethylamine (1). The ethereal solutions of the N,N,N′,N′-tetraethylisophthalamide and N,N,N′,N′-tetraethylterephthalamide were washed with an aqueous solution of sodium carbonate, and the amide was recovered on evaporation of the ether. The amide was then dissolved in chloroform and this solution was washed with a dilute aqueous solution of hydrochloric acid. The chloroform solution was then dried over anhydrous sodium sulfate and the amide was recovered on evaporation of the chloroform. The amides were then purified by recrystallization.

Reagents.—N,N,N′,N′ - Tetraethylfumaramide, m. p. 88°;[1] N,N,N′,N′-tetraethylisophthalamide, m. p. 85°; N,N,N′,N′-tetraethylterephthalamide, m. p. 134°; *p*-hydroxybenzoic acid, m. p. 216°; salicylic acid, m. p. 162°; chloramphenicol, m. p. 151–152°; 0.1 *N* alcoholic potassium hydroxide solution; bromothymol blue as indicator.

p-Hydroxybenzoic Acid.—Excess quantities of the acid (0.300 Gm.) were placed in rubber-stoppered vials together with varying quantities of the amide being studied and 10 ml. of 0.005 *N* sulfuric acid solution. The dilute acid solution was used to suppress any ionization of the *p*-hydroxybenzoic acid. The bottles were agitated in a constant temperature bath at 30° for fourteen hours. Aliquot portions of the supernatant liquid were then removed and analyzed for acid content by titration with 0.1 *N* alcoholic potassium hydroxide solution, with bromothymol blue as the indicator.

Salicylic Acid.—The experimental procedure was identical to that described for the *p*-hydroxybenzoic acid studies, with the exception that the quantity of acid used was 0.100 Gm.

Chloramphenicol.—An excess quantity of chloramphenicol (0.100 Gm.) was placed in 10-ml. rubber-stoppered vials together with varying quantities of the amide to be studied and 10 ml. of distilled water. The bottles were than agitated in a constant temperature bath at 30° for fourteen hours. Aliquot portions of the supernatant liquid were then removed and the chloramphenicol concentration was determined spectrophotometrically at a wavelength of 300 mμ.

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[1] All melting points uncorrected.

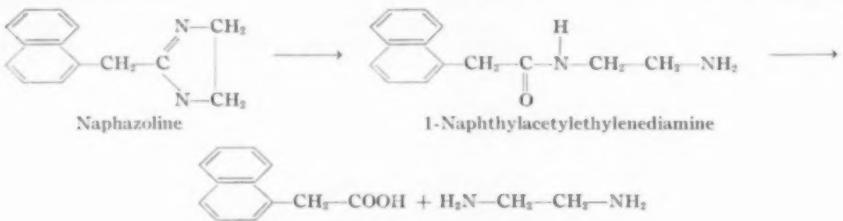
Chromatographic Separation and Analysis of Naphazoline and Its Hydrolytic Degradation Products*

By MICHAEL A. SCHWARTZ†, ROY KURAMOTO‡, and LOUIS MALSPEIS

The probable products of hydrolysis of naphazoline have been isolated. These have been separated by partition chromatography and subsequently analyzed spectrophotometrically. Quantitative recovery of these compounds has been obtained in assays of mixtures of known composition.

In order to study the kinetics of hydrolysis of naphazoline it was necessary to develop a quantitative assay procedure for the probable degradation components in solution after various time intervals.

Chemically naphazoline is 2-(1-naphthylmethyl)-2-imidazoline. The 2-imidazolines, being cyclic amidines, are generally subject to hydrolysis with the formation of 1,2-diamines. It has been shown (1) that naphazoline is quite stable in acid solution even after prolonged heating but it hydrolyzes in water or alkali to yield 1-naphthylacetylethylenediamine. It seemed likely that this compound, having an amide linkage, would undergo further hydrolysis to 1-naphthylacetic acid and ethylenediamine. The postulated overall reaction might therefore be represented as follows:



For purposes of a kinetic study of this system it would be desirable to know at any time interval during the course of the reaction, not only the concentration of residual naphazoline, but also the concentration of 1-naphthylacetylethylenediamine and of either 1-naphthylacetic acid or ethylenediamine.

A survey of the literature revealed several analytical procedures for some of these components (2–5). However, all of these were found to be inadequate since they would not distinguish between two or more of the degradation components.

Attempts at direct spectrophotometric analysis were unsuccessful because of the great similarity in absorption spectra of all the components except ethylenediamine.

Since a method for analysis of the hydrolysis products in the mixture was not available, they were separated by partition chromatography.

EXPERIMENTAL

Materials—Naphazoline hydrochloride,[1] m. p. 255–260°; 1-naphthylacetic acid, Fisher, highest purity, m. p. 131–133°; ethylenediamine, Mathieson, 98–100% pure; silicic acid, Mallinckrodt, chromatography grade; Celite® 545;[2] chloroform, U. S. P.; *n*-heptane; *n*-butanol, Baker analyzed reagent; ethyl acetate, Baker analyzed reagent; buffer solution, *p*H 8.5, 75 ml. 0.2 *M* boric acid plus 25 ml. 0.2 *M* sodium borate.

Equipment.—Beckman Spectrophotometer, Model DU; chromatograph tubes, 400 mm. long, 20 mm. in diameter, constricted at one end into a short delivery tube 7 mm. in diameter. Glass indentations were made near the lower end of the tube to support the column.

Preparation of 1-Naphthylacetylethylenediamine.—This was prepared by a modification of the method used by Miescher, *et al.* (1). Five grams of naphazoline hydrochloride was refluxed with 100 ml. of

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[1] Supplied by Ciba Pharmaceutical Products, Inc., Summit, N. J.
[2] Supplied by Johns-Manville, Lompoc, Calif.

0.5 *N* sodium hydroxide solution for thirty minutes. The resulting mixture was cooled, made strongly alkaline with concentrated sodium hydroxide solution, and then extracted with chloroform. The chloroform extract was evaporated to dryness leaving a yellowish oil which, upon chilling, gave an off-white colored solid. This was recrystallized several times from 1:1 chloroform-petroleum ether, m. p. 93–94.5°; reported m. p. 90–92° (recrystallized from alcohol-ether).

Anal.—Calcd. for $C_{14}H_{16}N_2O$: C, 73.65; H, 7.06; N, 12.27. Found:[3] C, 73.43; H, 7.14; N, 12.08.

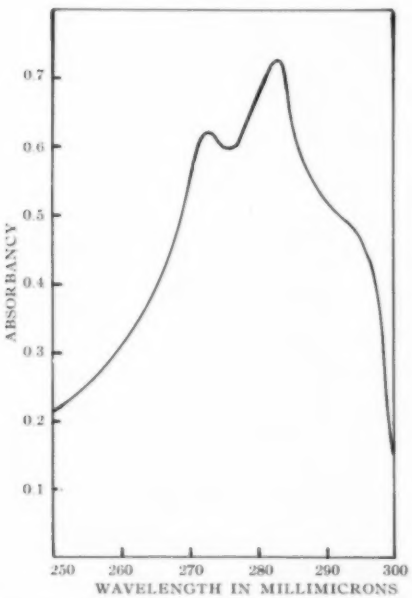

Fig. 1.—Ultraviolet absorption spectrum of 1-naphthylacetic acid in chloroform (0.020 Gm./L.).

The hydrochloride was prepared by dissolving the base in a small amount of chloroform and bubbling in anhydrous hydrogen chloride gas, which produced an oily liquid. As the chloroform was evaporated off, an off-white colored solid precipitated out. This was recrystallized from alcohol-anhydrous ether, m. p. 142–148°; reported m. p. 146–148°.

Anal.—Calcd. for $C_{14}H_{17}N_2OCl$: C, 63.16; H, 6.47; N, 10.58. Found:[3] C, 62.54; H, 6.67; N, 10.26.

Isolation of 1-Naphthylacetic Acid as a Hydrolysis Product.—Five grams of naphazoline hydrochloride was refluxed with 50 ml. of 1 *N* sodium hydroxide solution for two hours. This was cooled and acidified with hydrochloric acid giving a flocculent white precipitate which was filtered off,

[3] The authors wish to acknowledge the valuable assistance given by Mr. Dorfman and his group at Ciba Pharmaceutical Products, Inc., for performing these analyses.

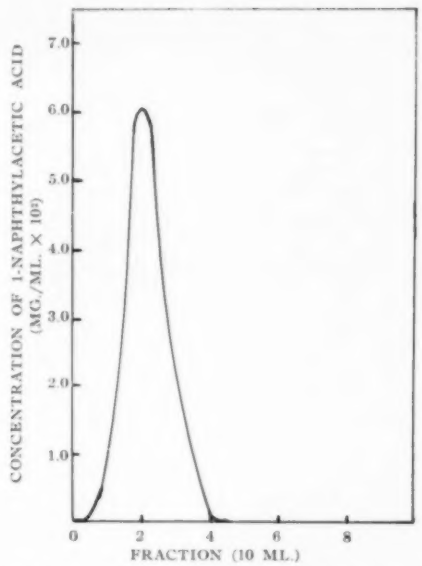

Fig. 2.—Chromatogram showing elution of 1-naphthylacetic acid from a silicic acid partition column.

washed with cold water, and recrystallized from hot water, m. p. 133–134°; reported m. p. of 1-naphthylacetic acid is 131–134°. A mixed melting point taken with a pure sample of 1-naphthylacetic acid was found to be 131–133°. Neutral equivalent found: 193.2; calcd. 186.2.

Partition Chromatography with Silicic Acid

Preparation of Column.—Fifteen grams of silicic acid was hydrated with 15 ml. of water in a 400-ml. beaker and sufficient chloroform added to make a thin slurry. The lower orifice of the chromatograph tube was stoppered, a plug of glass wool placed in the column, and a small amount of chloroform added. The slurry was then transferred to the tube in small portions, each portion being packed down tightly with a close-fitting glass plunger. A layer of solvent was always kept above the column to avoid entrapment of air.

Analysis.—Two grams of silicic acid was hydrated with exactly 2 ml. of the sample solution in a 30-ml. beaker. Chloroform was added to make a thin slurry and this was transferred to the column quantitatively. The beaker was rinsed with two 5-ml. portions of chloroform and the rinsings added to the column. The solvent was allowed to pass into the column before more solvent was added to elute 1-naphthylacetic acid. The first 100 ml. of eluate was collected in a volumetric flask and the concentration of 1-naphthylacetic acid determined spectrophotometrically.

The ultraviolet absorption spectrum of 1-naphthylacetic acid in chloroform is shown in Fig. 1. The compound exhibits maximum absorption at 283 mμ with $E(1\%, 1\text{ cm.})$ equal to 360.

Figure 2 shows the elution of 1-naphthylacetic

acid from the silicic acid partition column. The compound is eluted within the first 60 ml. of eluate while naphazoline and 1-naphthylacetylethylenediamine are held on the column.

Partition Chromatography with Celite 545

Preparation of Column.—Twenty grams of Celite 545 was covered with 175 ml. of 65:35 *n*-heptane-chloroform (v/v) in a 400-ml. beaker. To this, 15 ml. of *p*H 8.5 borate buffer solution was added dropwise with constant stirring to form a uniform slurry. This was then transferred to the chromatograph tube in the same manner as described in the preparation of the silicic acid column. A layer of solvent was always kept above the column to avoid entrapment of air.

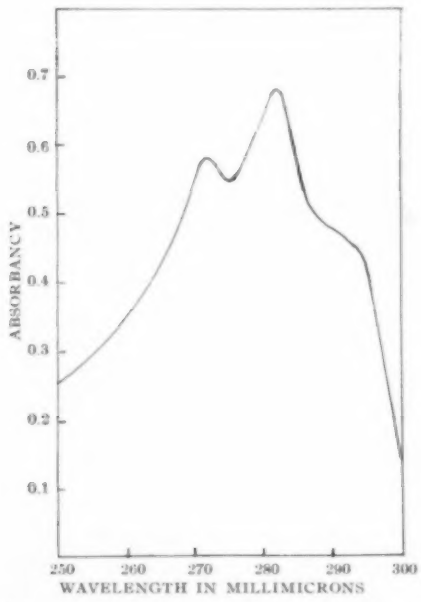

Fig. 3.—Ultraviolet absorption spectrum of naphazoline in 65:35 *n*-heptane-chloroform (v/v) (0.020 Gm./L.).

Analysis.—When all of the organic solvent had just entered the column, exactly 2 ml. of sample solution was pipetted on to the column. Then 65:35 *n*-heptane-chloroform (v/v) was added as eluant as soon as the sample passed into the column. The first 70 ml. of eluate was rejected. The next 125 ml., containing the naphazoline, was collected in 100-ml. and 25-ml. volumetric flasks and analyzed. The eluant was then changed to 100% chloroform and the first 10 ml. of eluate was rejected. The next 100 ml., containing the 1-naphthylacetylethylenediamine was collected in a volumetric flask and analyzed. The concentration of solute in the collected portions was determined spectrophotometrically at the wavelength of maximum absorption of solute.

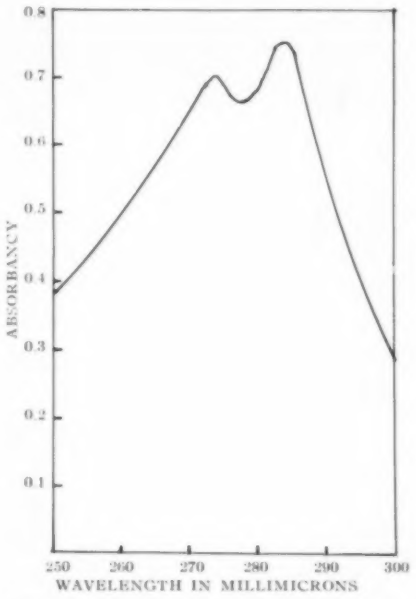

Fig. 4.—Ultraviolet absorption spectrum of 1-naphthylacetylethylenediamine in chloroform (0.020 Gm./L.).

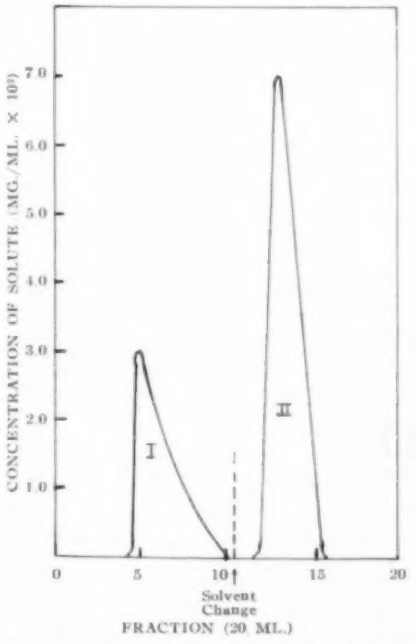

Fig. 5.—Chromatogram showing elution of naphazoline (I) and 1-naphthylacetylethylenediamine (II) from Celite 545 partition column.

The ultraviolet absorption spectrum of naphazoline in 65:35 *n*-heptane-chloroform (v/v) is shown in Fig. 3. The compound shows maximum absorption at 282 mμ with $E(1\%, 1\text{ cm.})$ equal to 340. The ultraviolet absorption spectrum of 1-naphthylacetylethylenediamine in chloroform is shown in Fig. 4. This shows maximum absorption at 284 mμ $E(1\%, 1\text{ cm.})$ equal to 314.

Since ethylenediamine does not absorb in this region it was not considered in the separation.

Figure 5 shows the elution of naphazoline and 1-naphthylacetylethylenediamine from the Celite 545 partition column.

RESULTS

Several solutions containing known amounts of the degradation products were prepared and analyzed by the procedures described. The results are shown in Table I.

TABLE I

Sample	Component[a]	Amount in Sample, mg.	Amount Found, mg.	Recovery, %
1	A	2.000	2.025	101.3
	C	1.000	0.993	99.3
2	A	1.707	1.713	100.4
	C	1.000	0.989	98.9
3	A	1.000	0.990	99.0
	B	1.000	0.973	97.3
	C	1.000	0.985	98.5
4	A	1.707	1.660	97.3
	B	2.000	2.066	103.3
	C	2.000	2.002	100.1

[a] Components are designated as: A, naphazoline; B, 1-naphthylacetylethylenediamine; C, 1-naphthylacetic acid.

It is apparent from the data in Table I that the precision of the method is within ± 3%.

SUMMARY AND CONCLUSIONS

1. Naphazoline hydrolyzes, upon heating in neutral or alkaline solution, to 1-naphthylacetylethylenediamine, which probably, upon further heating, breaks down to 1-naphthylacetic acid and ethylenediamine.

2. A two-step procedure for separating the components of the hydrolysate by means of partition chromatography has been developed. By partition with a 15-Gm. silicic acid column, containing 15 ml. of water as the internal phase, and using chloroform as the eluant, 1-naphthylacetic acid was separated from the other degradation components and analyzed spectrophotometrically.

3. Naphazoline and 1-naphthylacetylethylenediamine were separated by partition chromatography with a 20-Gm. Celite 545 column containing 15 ml. of *p*H 8.5 borate buffer solution as the internal phase. Naphazoline was eluted first using 65:35 *n*-heptane-chloroform as eluant. The solvent was then changed to 100% chloroform to quickly elute the 1-naphthylacetylethylenediamine. The concentration of solute in these eluates was determined spectrophotometrically.

Quantitative recovery of the degradation products was obtained in assays of mixtures of known composition using the methods described.

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A Note on Chemical Constitution and Biological Activity of Some Derivatives of Thiophosphoric Acid*

By M. L. BORKE and E. R. KIRCH

DURING RECENT YEARS certain organophosphorus compounds have come into use as insecticides and fungicides, while others are classified as "nerve gases." Their general formula may be represented as follows:

$$(R'O)_2\overset{\uparrow}{\underset{O(S)}{P}}—R''$$

The phosphoryl (P→O) or the thiophosphoryl (P→S) group present in all these compounds seems to be responsible for their affinity toward certain enzymes and particularly toward acetylcholinesterase (AChase).

In the course of an investigation of some derivatives of thiophosphoric acid, two series of compounds having the general formula

$$(R_1O)_2\overset{\uparrow}{\underset{S}{P}}—R_2$$

were prepared in this laboratory. In one of the series R_1 = C_6H_5, while in the second series R_1 = p-$C_6H_4CH_3$. In both series R_2 was an amine (aniline, *ortho-*, *meta-* or *para*-toluidine,

* Received June 27, 1956, from the Department of Chemistry, College of Pharmacy, University of Illinois, Chicago.

ortho- or *para*-anisidine and piperidine) or chlorine. All compounds are white crystalline solids, insoluble in water but soluble in ethanol.

The substances were tested for AChase inhibition *in vitro* by the Hestrin colorimetric method (1). A commercial preparation of AChase (Winthrop Laboratories) in a suitable medium was used as the substrate. Of the fifteen compounds tested only diphenyl chlorothiophosphate and di-*p*-cresyl chlorothiophosphate exhibited some inhibition of AChase. Their molar concentrations for 50 per cent inhibition of the enzyme (IN_{50}) were, respectively, 2.30×10^{-4} and 2.60×10^{-4}. None of the thiophosphoramides were inhibitory toward AChase.

The currently accepted theory (2) states that a basic group of the enzyme attacks the electrophilic phosphorus atom resulting in the transfer of the phosphoryl group to the basic site of the enzyme and yielding an inhibited enzyme of the structure (3):

$$(RO)_2\overset{\overset{G+}{\|}}{P}{-}O^- \longleftrightarrow (RO)_2\overset{\overset{G}{|}}{P}{=}O$$

Similar behavior can be expected of a thiophosphoryl group. Thus the more electrophilic the phosphorus atom, the stronger its tendency to combine with the basic site of the enzyme and the more powerful should be its inhibitory action, i.e., diisopropyl fluorophosphate (DFP) is a more powerful AChase inhibitor than its chloro-analog (4, 5) because of the stronger electrophilic tendencies of the phosphorus atom in the fluoro-compound.

If fluorine or chlorine (acyl groups) is replaced by other groups of acid character, equally potent AChase inhibitors are obtained. Examples of this class of compounds are diethyl-(4-nitrophenyl) phosphate (E 600, Mintacol) and diethyl-(4-nitrophenyl) thiophosphate (E 605, Parathion).

It has been observed that when R′ is an unsubstituted benzene ring the compounds are nearly inactive as compared to those in which the aromatic nucleus is substituted in *ortho* or *para* position by electron withdrawing groups (6). Compounds having the P→O grouping are more active than their sulfur analogs (7), and finally when R″ = p-$NHC_6H_4NO_2$ or $-N(R)_2$ the decrease in inhibitory activity toward AChase was also observed.

The results obtained in this laboratory seem to be in agreement with the currently accepted theory about the relationship between the chemical structure and the AChase inhibiting property of certain organophosphorus compounds.

This investigation is being continued.

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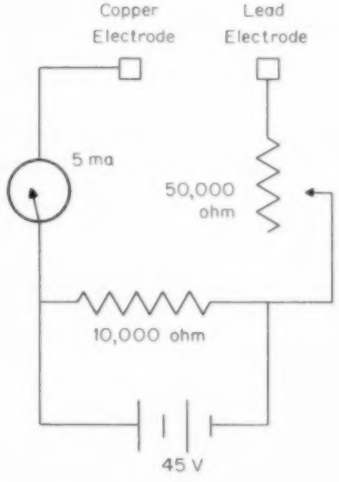
A Note on an Improved Iontophoretic Device*

By KEITH F. DENNISON, JOHN W. SCHERMERHORN, and WILLIAM H. BARGH

THE DEVELOPMENT of an iontophoretic device for producing histamine wheals on human subjects is reported by M. L. Cohen, *et al.* (1). In the course of an investigation in this laboratory it became necessary to produce such wheals. Because several preparations were to be tested and in order to speed the testing procedure, a modification of Cohen's apparatus was constructed. The circuit as shown in the figure includes a variable resistor of 0 to 50,000 ohms and a fixed resistor of 10,000 ohms to protect the milliammeter from damage. Current is provided by a 45-volt B battery.

It was found that a steady current of 1 milliampere could be delivered using as many as four electrodes attached to each terminal and that wheal production was obtained from exactly the same histamine concentration as was the case when only one electrode on each terminal was used.

The apparatus described is inexpensive and can be easily constructed. Experience in this laboratory indicates it is adaptable to both research and undergraduate instruction.



* Received June 6, 1956, from the Massachusetts College of Pharmacy, Boston.

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Book Notices

Catalysis. Vol. 4. By PAUL H. EMMETT. Reinhold Publishing Corporation, New York, 1956. vi + 570 pp. 15.5 x 23.5 cm. Price $12.50.

This 4th volume is of particular interest to the petroleum and coal industries. It is devoted entirely to the Fischer-Tropsch synthesis, and covers such important topics as the hydrogenation of carbon monoxide and related reactions, methanation catalytic hydrogenation of coal and tar, and catalytic cyclization and aromatization of hydrocarbons. The first two volumes in this series cover all of the general approaches and the tools used to gain a better understanding of both homogeneous and heterogeneous catalysis. Volume 3 summarizes existing knowledge on hydrogenation and dehydrogenation. These have been described more in detail in THIS JOURNAL, **43,** 446(1954) and **44,** 320 and 777(1955), respectively.

Other volumes, now in preparation, will cover the mechanics of reduction by hydrogen, oxidation, hydration, dehydration, halogenation, isomerization, alkylation, polymerization, cracking, decomposition, and many miscellaneous reactions. Chemists engaged in many different fields of research will find this series usually valuable for reference to all phases of catalytic processing.

Biology of the Laboratory Mouse. Edited by GEORGE D. SNELL. Dover Publications, Inc., New York, 1956 (Reprint of first edition, 1941). viii + 497 pp. 16 x 24.5 cm. Price $6.

This book compiled by the staff of the Roscoe B. Jackson Memorial Laboratory was originally published in 1941. Because the first edition appeared at the beginning of World War II it never attained widespread publicity or circulation. Despite this, it deserves attention as a standard guide for biologists, biochemists, bacteriologists, pharmacognosists and others who have occasion to use laboratory mice in experimental work. The scope of the book can best be indicated by listing the titles of the thirteen chapters: the early embryology of the mouse; reproduction; histology; spontaneous neoplasms in mice; gene and chromosomes mutations; the genetics of spontaneous tumor formation; the genetics of tumor transplantation; endocrine secretion and tumor formation; the milk influence in tumor formation; inbred and hybrid animals and their value in research; parasites; infectious diseases of mice; and care and recording.

The publishers have reprinted the 1941 edition and the purpose of this description is to indicate that the book is available.

The Examination of New Organic Compounds. By WALTER T. SMITH, JR. and RALPH L. SHRINER. John Wiley and Sons, Inc., New York, 1956. vii + 136 pp. 21.5 x 29 cm. Price $3.50.

This paper-bound laboratory manual is designed to furnish information useful in orienting seniors and graduate students in research in organic chemistry. It contains adequate descriptions of methods for the quantitative analysis and characterization of organic compounds. Semimicro methods are liberally employed, but macromethods are not neglected. The methods described have been selected from numerous articles in the literature and rewritten and modified so as to be of maximum usefulness to students inexperienced in analytical and characterization methods. References to the original literature are given at the end of each chapter. The manual is divided into five parts covering general principles; analysis for elements; molecular weight determinations; functional group analysis; and problems. The book is further subdivided into thirty-nine chapters and a functional subject index is provided. This new laboratory manual should serve adequately as an introduction to an important phase of training for research in organic chemistry.

Modern Chemical Processes. Vol. 4. By the editors of Industrial and Engineering Chemistry. Reinhold Publishing Corporation, New York, 1956. v + 202 pp. 21 x 29 cm. Price $5.

This book is the fourth volume in a series prepared under the auspices of the editors of Industrial and Engineering Chemistry. It contains nineteen chapters, each of which is contributed by authors closely associated with the subjects covered. Recently developed chemical processes are described, beginning with their commercial-and technical history and followed by descriptions of plant installations, process and operation of plants, and future prospects. This volume, as well as those which preceded it, will appeal especially to the practicing industrial chemist and the chemical engineer.

The Chemistry and Technology of Waxes. New 2nd ed. By ALBIN H. WARTH. Reinhold Publishing Co., New York, 1956. vii + 940 pp. 16 x 23.5 cm. Price $18.

The first edition of this book was published in 1947. Since then there have been many developments in wax research and technology. As a result, the second edition has been greatly expanded and includes complete up-to-date information on the entire wax industry from the natural to the applied sciences. This information is supplemented by many new illustrations, charts, tables, and literature and patent references. Chemists and others in the pharmaceutical and cosmetic industries who require a knowledge of waxes in their work will find this book an excellent reference source. It brings together and collates much valuable information not otherwise obtainable except by an intensive search of the original literature. The book should also appeal to teachers of pharmacy and graduate students engaged in research leading

to the development of new pharmaceutical preparations requiring the use of waxes in formulations.

Every Other Bed. MIKE GORMAN. The World Publishing Company, New York, 1956. 318 pp. 15 x 22 cm. Price $4.

The status of mental illness in the United States, told in hard-hitting style, will shock the unacquainted reader, but leave him with a feeling of great hope for the future and a desire to participate in accelerating research for control of this common affliction. Mike Gorman, an experienced newspaperman, and author of "Oklahoma Attacks Its Snake Pits," has devoted a great many years in a crusade toward bettering the lot of the mentally ill. As Executive Director of the National Mental Health Committee, he is in a strategic position to assay the medical, financial, and legislative necessities in tackling the mental health problem aggressively. This volume, using numerous direct quotations from the writings, speeches, and statements of recognized medical authorities, dramatically portrays the plight of the mental patient, the conditions in mental institutions, the paucity of funds for mental health research and the recent breakthrough of new drugs. Many readers will be surprised to learn that every other bed in U. S. hospitals is occupied by a mentally ill patient, and most will be appalled at the two and a half billion dollar annual cost of mental illness. Mr. Gorman does a fine job of presenting the facts and making his point that a great team effort on a broad front will bring results. New drugs like Reserpine and Chlorpromazine already are reducing the population of patients in mental hospitals. This is the start of a new era in the field of mental health and Mr. Gorman bids us as a nation to roll up our sleeves and get behind a strong research effort to accelerate progress. *Every Other Bed* is interesting, challenging, easy reading. Its popular style will appeal to the reader, who will come away with a feeling of having been educated in the complexities of mental disease problems. This volume will do much to spur support of medical research on mental diseases. It can be recommended for general reading.

Directory American Council of Independent Laboratories, Inc. 6th ed. American Council of Independent Laboratories, Inc., Washington, D. C., 1956. xxxi + 67 pp. 17.5 x 26 cm.

This Directory lists thirty members of the American Council of Independent Laboratories who specialize in drug and pharmaceutical industry problems. They provide services in research, consultation, analyses, and product development and improvement. In analyses, U. S. P., N. F., and A. O. A. C. tests are used where applicable. Members of the Council employ some 2000 chemists, biochemists, pharmacologists, bacteriologists, and engineers. Clients of independent laboratories retain complete control over patents and publication of scientific findings. The formal written report given the client becomes his exclusive property. Confidential information obtained from previous clients is not used to their detriment.

Czechoslovak Fine Chemicals Standards. Vol. 1, M. NEDELJAK, editor. Chemapol, Praha 11, Czechoslovakia, 1955. 540 pp. 12 x 21.5 cm.

This book, written in English, is published by a foreign trade corporation for the import and export of chemical products and raw materials. The standards were prepared by a committee of the Czechoslovak Standard Institution, a government agency. It is designed to furnish standards for fine chemicals used in Czechoslovakia. In general, only the simplest methods of testing have been used in order to enable smaller laboratories to determine the quality of chemicals. The standards were adopted only after endorsement by approximately 100 consumers in manufacturing, university, and research institute laboratories. Three grades of chemicals are recognized. Guaranteed Reagent Special (GRS) is the purest quality that may be required for special scientific work. This quality is produced on special request only. Another quality, Guaranteed Reagent (GR), is designed for usual analytical procedures where an especially high grade of purity is required. A third grade of purity is indicated in the standards as Pure (P), for chemicals not requiring as high a degree of purity as those in the other two categories. The book includes 144 monographs giving specifications for various types of reagents and test solutions. Most monographs are divided into five parts covering terminology, general notes, technical requirements, tests, and storage requirements. Descriptions of test procedures are given in detail in each monograph. Specifications for three grades of the same chemical are furnished for only a limited number of the chemicals for which standards are given. For the most part, specifications are furnished for the more widely used reagents, but formulas and functional tests are supplied for a number of clinical laboratory reagents such as Pandy's, Schlesinger's, and Schweitzer's solutions. Also included are standards for some of the rare sugars such as cellobiose, D-erythrose, D-lyxsose, D-ribose, D-tagatose, and trehalose. The assay procedures and limit tests for trace impurities are comparable to those found in pharmacopeias and publications of other countries relating to standards for reagent chemicals.

The Condensed Chemical Dictionary. 5th ed. By ARTHUR and ELIZABETH ROSE. Reinhold Publishing Corporation, New York, 1956. xix + 1200 pp. 16 x 23 cm. Price $12.50.

This revised and enlarged edition of an exceptionally useful reference for those interested in chemistry and related fields is as up-to-date as any published volume can be in this category. The extensive coverage of medicinal and pharmaceutical chemicals and materials makes the book useful to pharmacists. Many trademark names are included in the entries. Numerical and alphabetical lists of manufacturers are given and the manufacturer's number is included after each trademark name. The editors condense much useful information under the many entries in this expanded dictionary. The book should be in all libraries.

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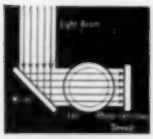
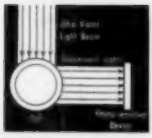


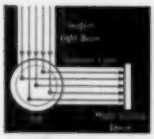
AS A COLORIMETER

Light of specified wavelengths is absorbed by solution. Amount absorbed is measured and related to concentration.



AS A FLUOROMETER

Light of one wavelength causes some substances to fluoresce. The fluorescent light is measured and related to the concentration.



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